MECHANISMS OF ION CHANNEL ACTIVATION IN OLFACTORY RECEPTOR CELLS OF THE SPINY LOBSTER, PANULIRUS ARGUS

By

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by

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TO MY PARENTS

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KEY TO ABBREVIATIONS

ADP = adenosine 5'-diphosphate

AMP = adenosine 5'-monophosphate

ATP = adenosine 5'-triphosphate

8-bromo-cAMP = 8-bromo-cyclic adenosine 3',5'-monophosphate

8-bromo-cGMP = 8-bromo-cyclic guanosine 3',5'-monophosphate

cAMP = cyclic adenosine 3',5'-monophosphate

cGMP = cyclic guanosine 3',5'-monophosphate

CNS = central nervous system

EGTA = ethylene glycol bis-N,N,N',N'-tetraacetic acid

f_c = corner frequency

GDP-β-S = guanosine 5'-O-(2-thiodiphosphate)

GTP = guanosine 5'-triphosphate

GTP-Γ-S = guanosine 5'-O-(3-thiotriphosphate)

HA = histamine

HEPES = N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid

I = current

IBMX = 1-isobutyl-3-methylxanthine

P_o = probability of an ion channel being in the open state

R = resistance

TEA = tetraethylammonium

TTX = tetrodotoxin

V = voltage

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Timothy S. McClintock

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As in any cell, ion channels underlie the active electrical properties of the lobster olfactory receptor cell. Studying the mechanisms of activation of these ion channels is a step toward understanding how the olfactory receptor cell performs its functions.

Somata isolated from olfactory receptor cells contained three voltage-dependent currents: a sustained Ca^{++} current, a Ca^{++}-activated K^+ current, and a delayed rectifier K^+ current. A TTX-sensitive Na^+ current occurred predominantly in the axon. Four ion channels were described: a 35 pS voltage-independent Cl^- channel, a 9.7 pS delayed rectifier K^+ channel, a 215 pS Ca^{++}-activated K^+ channel and a nonselective cation channel.

Radioimmunoassays of cAMP in preparations containing the outer dendritic segments of the receptor cells, where odor reception and transduction occur, revealed only a low level of adenylate cyclase

activity, and no significant changes were evoked by odorants. Intracellular recordings demonstrated that forskolin or cAMP analogs mixed with a phosphodiesterase inhibitor and applied to the outer dendritic segments did not evoke responses directly or cause the cell's odor response to adapt. These results do not support the hypothesis that cAMP mediates transduction in lobster olfactory receptor cells.

A mixture of L-arginine, L-proline, and L-cysteine evoked hyperpolarizing receptor potentials in both P. argus and the American lobster, Homarus americanus, providing an explanation for noncompetitive mixture suppression in odor responses. All cells hyperpolarized by this mixture were also depolarized by other odorants, indicating that multiple transduction mechanisms may exist in the same cell.

Histamine directly gated a chloride channel in receptor cell somata. This channel required external histamine for gating, but not GTP-binding proteins, metabolic energy sources, or Ca^{++}. The conductance of the channel was 66 pS in P. argus and 44 pS in H. americanus. The pharmacology and dose-dependency of this channel indicate that it mediates the suppression of excitability of the lobster olfactory receptor cell caused by histamine.

CHAPTER 1
GENERAL INTRODUCTION

Background

Sensory systems provide an animal with the environmental information necessary to make adaptive behavioral responses. An understanding of how sensory systems perform this function begins with the role of the receptor cell. Receptor cells must receive stimuli, transduce stimulus energy into generator potentials or action potentials, and transmit the electrical signals to the CNS. They may also participate in the filtering of excessive or adaptively insignificant signals. The molecular mechanisms underlying these processes must be understood because they determine which stimuli a sensory system is capable of detecting and how the stimulus is encoded into the neural signals which are the internal representation of an organism's environment. Receptor cells in the vertebrate visual system are the most thoroughly investigated examples (reviewed by Stryer, 1986). In photoreceptors, light activates rhodopsin, which activates transducin, which activates a phosphodiesterase. The phosphodiesterase catalyzes the hydrolysis of cGMP, which decreases the cGMP-activated inward current, and consequently decreases synaptic activity. This decrease in synaptic activity is then the signal which is processed by the retina and passed on to higher neural levels.

In comparison to other receptor cells, especially photoreceptors, the olfactory receptor cell is poorly understood. The non-spatial and physically discrete nature of olfactory stimuli increases the complexity of determining stimulus quality. Unlike vertebrate taste, where recognition of four basic qualities simplifies categorization of responses, and where recent evidence suggests that stimulus quality could be determined by distinct transduction mechanisms (reviewed by Kinnamon, 1988), olfactory responses appear to contain infinite variety. The unique features of olfaction provide an opportunity to investigate mechanisms of sensory coding, and ultimately perception, which may contrast with known mechanisms in unforeseen ways, potentially contributing to a more broadly based understanding of sensory coding.

Olfactory receptor cells detect and encode chemical signals. They provide an organism with information about its chemical environment, especially about low concentrations of odorants, often emanating from distant sources. The activation of olfactory receptor cells by odorants is a complex phenomenon which can be divided into 5 processes: the perireceptor events, the binding of odorants to receptors, olfactory transduction, the spread of odor-evoked currents, and the effects of these currents on the generation of action potentials. These processes have critical, interdependent roles in the activation of the receptor cell.

Perireceptor events involve proteins which facilitate the access of odorants to receptor sites, as well as proteins which help to clear the odorants away from the receptor sites, as occurs in

synaptic clefts. Odorant-binding proteins, which seem to carry odorants to, or concentrate odorants near, the receptor sites have been characterized in insects (Vogt, 1987) and mammals (Bignetti et al., 1985; Pevsner et al., 1985, 1988). Clearance systems such as degradative enzymes and uptake systems have been observed in both insect (Vogt et al., 1985) and mammalian olfactory organs (Dahl, 1988). In lobster olfactory organs, ectonucleotidases which degrade adenine nucleotides, and uptake systems for adenosine and taurine, have been described (Trapido-Rosenthal et al., 1987; Gleeson et al., 1987).

Once odorants reach the olfactory receptor cells, they are believed to bind to receptor proteins (reviewed by Lancet, 1988) which presumably reside in the membranes of the dendrites (Adamek et al., 1984). Binding studies using volatile odorants have proven difficult because these lipophilic odorants can bind nonspecifically to hydrophobic surfaces (reviewed by Lancet, 1988). But the binding of amino acids to chemosensory membranes of fish has provided evidence for receptor proteins (Cagan and Zeiger, 1978; Brown and Hara, 1981; Fesenko et al., 1983). Receptor proteins presumably also exist in lobster olfactory receptor cells because the identified odorants are amino acids and other small, water-soluble compounds (Carr et al., 1984; Derby and Ache, 1984a).

Olfactory receptor proteins activated by the binding of odorants must then generate an electrical response, a process called olfactory transduction. Evidence for several mechanisms of olfactory transduction now exist. Biochemical studies demonstrating

that volatile odorants stimulate adenylate cyclase in olfactory cilia from frogs and rats (Pace et al., 1985; Sklar et al., 1986) have received electrophysiological support. The perfusion of cAMP into salamander olfactory receptor cells evokes an inward current (Trotier and MacLeod, 1987), and both cAMP and cGMP activate a nonselective cation channel in excised patches from frog olfactory cilia (Nakamura and Gold, 1987). Application of 8-bromo-cAMP, forskolin, or phosphodiesterase inhibitors to voltage-clamped sheets of frog olfactory mucosa also gives results which are consistent with olfactory transduction by cAMP (Desimone et al., 1988). In contrast, amino acids stimulate phosphoinositide turnover in catfish olfactory cilia (Huque and Bruch, 1986), and a novel odorant-gated channel has been reported in recordings of ion channels from frog olfactory cilia reconstituted in planar bilayers (Labarca et al., 1988). Although this evidence is impressive, especially for cyclic nucleotides, proof that any of these mechanisms is necessary for olfactory transduction is still lacking.

Once transduction has generated an ionic current, the current must spread to interact with the voltage-dependent ion channels in the spike generating zone. Because olfactory receptor cells are bipolar neurons, odor-evoked currents must spread from the most distal sections of the dendrites to the axons where action potentials appear to be generated (Masukawa et al, 1985; Trotier, 1986; Schmiedel-Jakob, et al., 1989). Current spread is either passive, or active mechanisms (i.e., voltage-dependent channels) must participate. Present evidence from amphibian olfactory

receptor cells, including high membrane resistance and the apparent absence of voltage-dependent currents whose properties would allow them to enhance the spread of current (Firestein and Werblin, 1987; Trotier, 1986; Masukawa et al., 1985), is consistent with passive spread.

When the odor-evoked current reaches the spike generating zone, the resulting change in membrane potential will change the spike frequency. The pattern and frequency of these spikes presumably encode information about the quality and quantity of the odorants present (reviewed by Lancet, 1986). The characteristics of the spike trains generated in response to excitatory odorants, such as frequency adaptation and maximum and minimum spike frequencies, are functions of the voltage-dependent currents of the receptor cell and the underlying receptor potential. At present, our knowledge of voltage-dependent currents and the ion channels which underlie them in olfactory receptor cells is sketchy at best. Some differences in channels and currents in olfactory receptor cells occur between amphibian species (Trotier, 1986; Firestein and Werblin, 1987; Suzuki, 1987), and greater differences occur between amphibian and mouse olfactory receptor cells (Maue and Dionne, 1987). This suggests that a number of possible solutions exist for the problem of regulating the frequency of spike trains.

These activation processes may not always proceed without intervention from other parts of an animal's nervous system, however. Evidence that receptor cell excitability may be regulated by neuroactive substances suggests that the CNS may feed back onto

olfactory receptor cells. In frogs, substance P, perhaps released from the trigeminal system, affects the electrical activity of the olfactory receptor cells (Bouvet et al., 1987). In mosquitoes, blood-borne factors cause decreased sensitivity of extracellularly recorded responses to odorants (Davis and Takahashi, 1980). In the olfactory receptor cells of lobsters, histamine suppresses both spontaneous and odor-evoked spiking and decreases the amplitude of receptor potentials (Bayer et al., submitted). The mechanisms mediating these regulatory phenomena were unknown, until the evidence presented in Chapter 6 demonstrated that a ligand-gated channel mediates the action of histamine in lobster olfactory receptor cells.

The Olfactory Organ of the Spiny Lobster

An attractive system for studying the activation of olfactory receptor cells is the olfactory organ of the spiny lobster, Panulirus argus. The odorants which have been identified are amino acids, nucleotides, and other small water-soluble molecules (Carr et al., 1984; Derby and Ache, 1984a). Some of these molecules are neurotransmitters and neuromodulators, providing interesting comparisons with their known mechanisms of action at internal sites of chemoreception, such as synapses (Carr et al., 1987; 1989). The olfactory receptor cells of the spiny lobster have long been the focus of electrophysiological studies (reviewed by Ache and Derby, 1985). Extracellular recording studies have made significant contributions to our understanding of olfactory coding phenomena, such as mixture suppression (Derby and Ache, 1984a; Derby et al.,

1984; Gleeson and Ache, 1985; Ache et al., 1988) and discrimination of complex mixtures of odorants (Derby and Ache, 1984b; Girardot and Derby, 1988). The pioneering study of P. argus by Anderson and Ache (1985), was the first in which patch-clamp methods were used to obtain reliable intracellular recordings of odor responses from an olfactory receptor cell. With methods and preparations for intracellular and patch-clamp recording now available, the ability to study the electrical properties and mechanisms of activation of the olfactory receptor cells is enhanced.

A significant advantage of the olfactory organ of P. argus is its morphology. The primary olfactory organ consists of the aesthetasc sensilla located on the lateral filaments of the paired first antennae, or antennules (Fig. 1-1). The ultrastructure of these aesthetascs was recently described by Grünert and Ache (1988). An aesthetasc consists of a cluster of about 320 bipolar sensory neurons, each of which sends a dendrite into the 800 μm long cuticular hairs (Fig. 1-2). There may be up to 2000 aesthetascs and 600,000 olfactory receptor cells per olfactory organ. The dendrites of the receptor cells branch repeatedly into outer dendritic segments which extend into the distal 700 μm of the aesthetasc hair. The only other cellular material inside the hairs is the processes of the auxiliary cells which ensheathe the inner dendritic segments in the proximal 100 μm of the hair. The inner dendritic segments are 300 - 400 μm in length and are about 0.5 μm in diameter. The outer dendritic segments are 600 to 700 μm in length and 0.05 to 0.08 μm in diameter. In the base of the hair, septate junctions and

desmosomes between auxiliary cells, and desmosomes between auxiliary cells and inner dendritic segments, appear to prevent the free exchange of solutions between the hair lumen and the hemolymph space in the antennular lumen. Auxiliary cells also ensheathe the neuronal clusters in the antennular lumen. The axons run uninterrupted from the neuronal cell bodies to the olfactory neuropil of the CNS. This morphology provides several advantages. Dissection is made easy by the exposed location of the organ. By simply cutting off the hairs one can obtain an almost pure preparation of olfactory membranes presumably containing all the biochemical machinery necessary for the initiation of odor responses. The physical separation of the solutions bathing the outer dendritic segments and the soma allows selective application of different solutions to these parts of the cells.

Statement of Purpose

The first objective of this project was to establish a foundation of information about the ion channels and ionic currents in the olfactory receptor cell of the spiny lobster. Description of these channels and currents helped me to formulate several hypotheses about mechanisms involved in the spread of odor-evoked currents, olfactory transduction, inhibitory odor responses, and the regulation of excitability of the receptor cell. From these hypotheses I made four predictions.

The second objective was to test the predictions, which were as follows. (1) A weakly voltage-dependent ion channel carrying inward current enhances the spread of odor-evoked inward currents. (2)

Odorants stimulate adenylate cyclase to produce cAMP as a mechanism of olfactory transduction. (3) Hyperpolarizing receptor potentials exist. (4) A ligand-gated channel mediates the suppression of olfactory receptor cell excitability by histamine.

Fig. 1-1. A. A drawing of the spiny lobster showing the location of the aesthetasc sensilla on the lateral filaments of the antennule. B. Four annuli of the antennule. The shaved aesthetasc hairs on the first antennular section show the parallel arrangement of the bases of the double row of hairs before they bend into the zig-zag relationship formed by their tips. Reproduced from Grünert and Ache (1988), with permission.

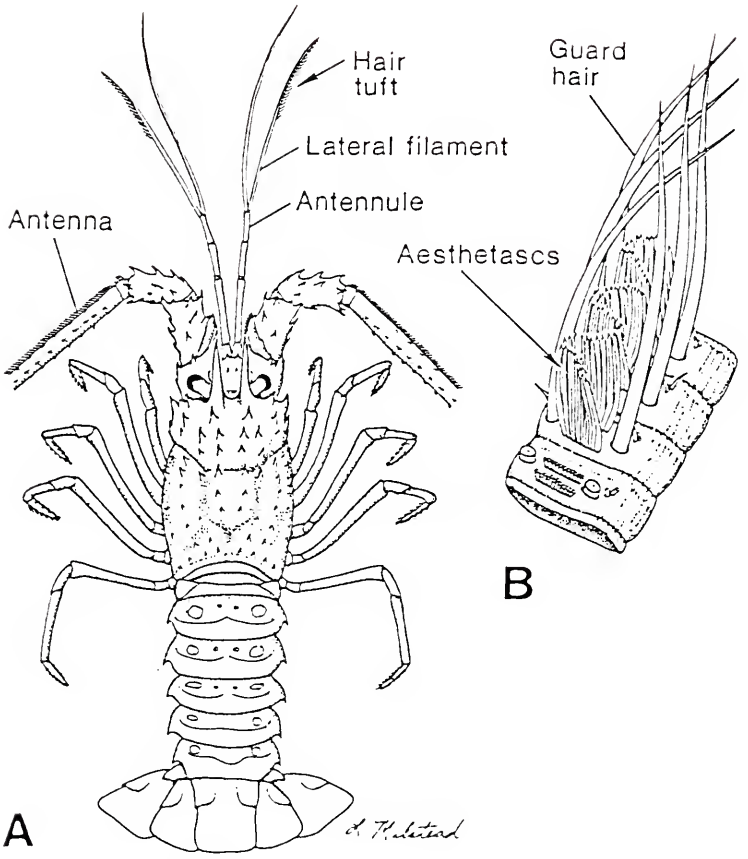
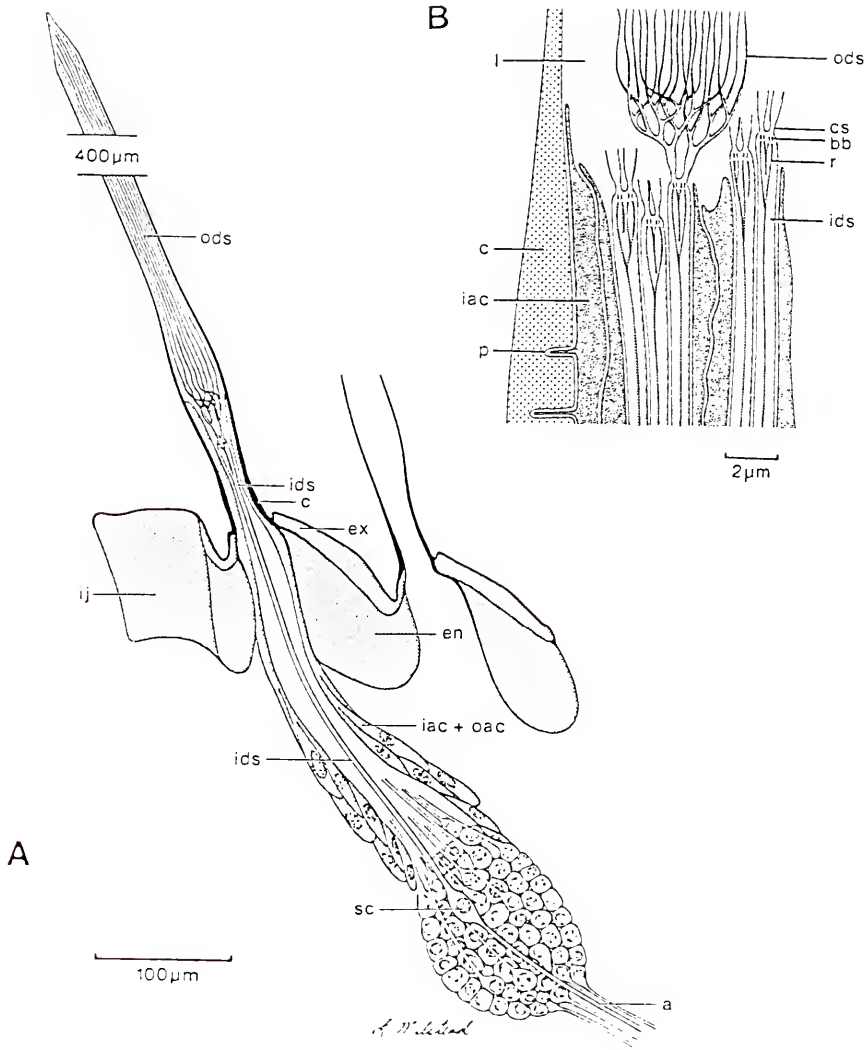

Hair
tuft
Lateral filament
Antennule
Antenna
A
Guard
hair
Aesthetascs
B

Fig. 1-2. The aesthetasc sensillum of Panulirus argus. A. A diagram of an aesthetasc sensillum in longitudinal section. The proportions of the structures inside the hair are not drawn to scale. B. A drawing of a longitudinal section of the region of the hair where the dendrites branch. Axon (a); basal body (bb); bulbous region (br); cuticle (c); ciliary segment (cs); endocuticle (en); exo- and epicuticle (ex); inner auxiliary cell (iac); inner dendritic segment (ids); intersegmental junction (ij); lymph space (l); outer auxiliary cell layer, (oac); outer dendritic segment (ods); pore-like structure (p); ciliary rootlet (r); sensory cell soma (sc). Reproduced from GrÜnert and Ache (1988), with permission.


B
l
ods
cs
bb
r
ids
c
iac
p
2µm
400µm
ods
ids
c
ex
ij
en
iac + oac
ids
A
sc
100µm
a

CHAPTER 2
IONIC CURRENTS

Introduction

Olfactory receptor cells in diverse organisms are primary bipolar sensory neurons that serve both to transduce the stimulus and generate action potentials that propagate to the CNS. Transduction is thought to occur in the terminal arbor of the dendrite, which is exposed to the odor environment (reviewed by Getchell and Getchell, 1987). Whole-cell patch clamp recordings in amphibians (Firestein and Werblin, 1987; Trotier, 1986) and crustaceans (Schmiedel-Jakob et al., 1989) indicate that olfactory receptor cells have a high membrane resistance, permitting odor-evoked receptor potentials to invade the soma and generate action potentials in or near the axon. These intracellular findings support similar interpretations of extracellular data from olfactory receptor cells in vertebrates (Getchell, 1973). Extracellular data from insect olfactory receptor cells are as yet inconclusive as to the site of origin of action potentials (reviewed by Kaissling, 1986; De Kramer and Hemberger, 1987). Because the soma is critically interposed between the transduction site and the output of the cell, its voltage-dependent ionic currents, as well as its passive membrane properties, play a critical role in determining spike output by providing voltage-activated responses such as

rectification or afterhyperpolarization. Furthermore, because the soma is usually the recording site and the injection site of drugs, the contribution of the soma to experimental manipulations designed to affect the dendrites must be considered. For these reasons, an understanding of the somatic ion channels and currents is important.

Analyses of the ionic currents in olfactory receptor cells have previously been confined to amphibians (Firestein and Werblin, 1987; Trotier, 1986; Suzuki, 1987). Collectively, amphibian olfactory receptor cells possess a transient Na^+ current, a Ca^{++} current, a delayed rectifier current, a transient outward current (A current), a Ca^{++}-activated K^+ current, and perhaps an anion current. As these currents were recorded from cells with intact dendrites, the role of channels in the somatic membrane is unclear. In order to properly interpret whole-cell recordings of odor-evoked currents, to better understand the spread of these currents within the cells, and to further the comparative perspective of olfactory receptor cell function, I have investigated the ionic currents of somata isolated from olfactory receptor cells of the lobster, P. argus.

This chapter describes the properties of four voltage-dependent currents from olfactory receptor cells. These currents are a TTX-sensitive Na^+ current, a Ca^{++} current, a delayed rectifier K^+ current, and a Ca^{++}-activated K^+ current. How these currents could produce the voltage-dependent responses of the olfactory receptor cell -- action potentials, membrane repolarization, outward rectification, and repetitive discharge of action potentials -- is discussed.

Methods

Preparation of isolated olfactory receptor cell somata

The lateral filament of an antennule (the olfactory organ) was excised from specimens of *P. argus*, and cut into 1 mm hemisections (Anderson and Ache, 1985). This procedure removes the majority of the axon from each soma. Hemisections were agitated with L-cysteine-activated papain (.25 mg/ml; Sigma, type IV) for 25 min and then with trypsin (2.5 - 5 mg/ml in Ca^{++} free saline; Sigma, type IX) for another 25 min. Somata were isolated and stripped of their dendrites by drawing the somata clusters into a glass pipette with a 200 µm opening and forcing them back out into a 35 ml plastic culture dish, where they were allowed to settle and stick to the bottom. Olfactory receptor cell somata were identified by their spherical shape and 10 - 20 µm diameter. An ultrastructural study found that virtually all cells of this size and shape in the olfactory organ are olfactory receptor cells (Grünert and Ache, 1988).

Voltage-clamp recording

Patch clamp pipettes were made from borosilicate glass tubing (Boralex, Rochester Scientific, Rochester, NY) pulled to a tip diameter of just over 1 µm (Bubble numbers of 4.6 to 5.4, Mittman et al., 1987). Sylgard 184 (Dow Corning, Midland, MI) was applied to the neck of the pipette to reduce capacitance. After forming a gigaohm seal and compensating the capacitance of the electrode, the whole-cell recording configuration was achieved using suction to break through the cell membrane and the series resistance error was

compensated qualitatively. A commercial voltage-clamp and current amplification circuit with a 1 gigaohm head stage (Dagan Instr. model 8900, Minneapolis, MN) was used to record macroscopic currents from the somata. An IBM-XT computer with a D/A, A/D converter and accompanying software (pClamp, Axon Instr., Burlingame, CA) was used to apply voltage-step protocols and to store digitized data for later analysis. Data were low-pass filtered at a corner frequency of 10 kHz. Leak currents were recorded from each soma by applying half amplitude voltage step protocols at hyperpolarized potentials where no active currents were elicited. Leak correction was then performed by multiplying these leak currents by $\pm$ 2 as necessary, and subtracting them from the active current records. Recordings were made at room temperature, which varied from 22 to 24°C.

Solutions

The composition of lobster saline was (mM) 480 NaCl, 13 KCl, 13 $CaCl_2$, 10 $MgCl_2$, 1.7 glucose, 20 HEPES, pH 7.4. The composition of the normal patch solution used was (mM) 200 KCl, 2 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 570 glucose, 10 HEPES, 34 NaOH, pH 7.4. The compositions of other solutions used are described in the figure legends. Inorganic salts were purchased from Fisher Scientific (Fair Lawn, NJ) and HEPES and EGTA were purchased from Research Organics (Cleveland, OH). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

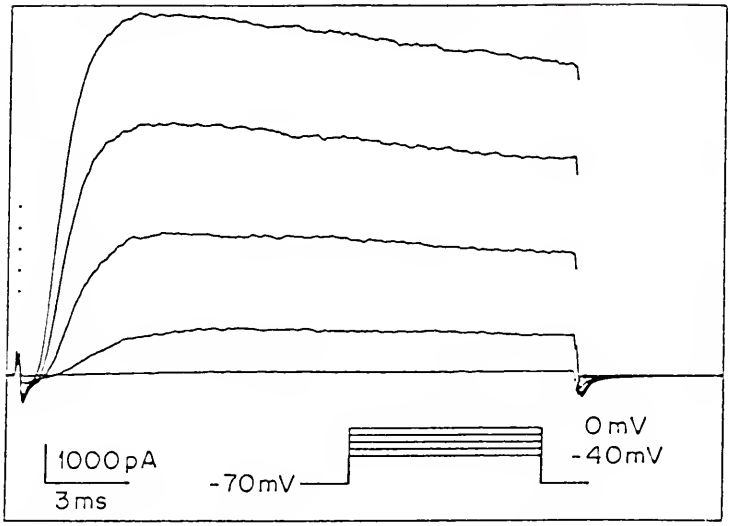
Total membrane currents

In response to depolarizing voltage steps, isolated somata bathed in lobster saline and perfused with normal patch solution showed an inward current, followed by a much larger outward current (Fig. 2-1). Hyperpolarizing voltage steps down to -120 mV, from holding potentials of -35 to -70 mV, failed to activate any currents in these cells. The inward current became apparent at slightly more negative potentials (-40 to -30 mV) than did the outward current (-30 to -20 mV). Specific blocking agents and impermeable ions were then used to isolate four component currents from the total membrane currents of these somata. In no instance did a cell fail to exhibit the appropriate current for the recording conditions used.

Na^+ current

Replacing the K^+ in the patch solution with Cs^+ and adding 4 mM Co^{++} or Cd^{++} to the bath allowed isolation of a fast, transient inward current (N = 24 cells) that peaked within 1 ms of the initiation of the voltage step (Fig. 2-2). This current was absent in the presence of submicromolar concentrations of TTX (N = 111 cells) and saxitoxin (N = 2 cells), identifying this as a Na^+ current. The current activated at -38 to -30 mV and reached maximum amplitude at 0 to 10 mV. The rapid onset of the current at -6 mV was described by a single exponential with a time constant of 0.07 ± 0.03 ms (N = 7 cells). The current decayed as a single exponential function with a time constant of 0.29 ± 0.08 ms (N = 13 cells). Large Na^+ currents were not correlated with cell size as measured by

Fig. 2-1. Total membrane currents of an isolated soma. Above: Macroscopic currents evoked by depolarizing voltage steps (inset) showing a small, biphasic inward current and a large outward current. Leak currents were not subtracted from these records. Below: The I-V relationships of the inward current (circles) and the outward current (triangles) from this cell. Pipette: normal patch solution. Bath: saline.


1000pA
3ms
-70mV
0mV
-40mV

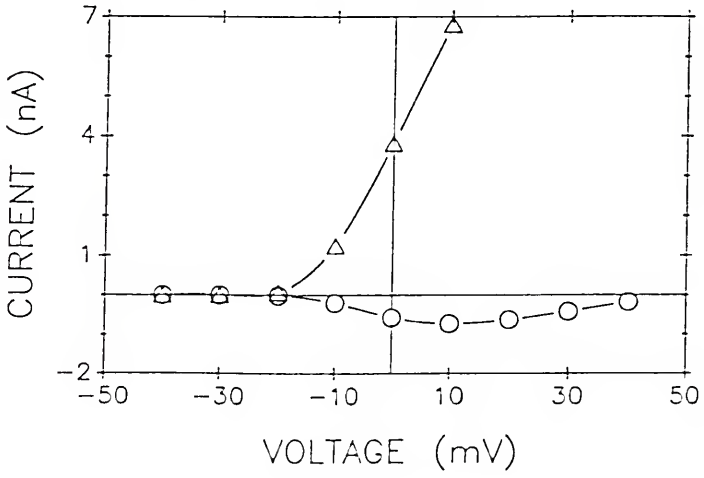
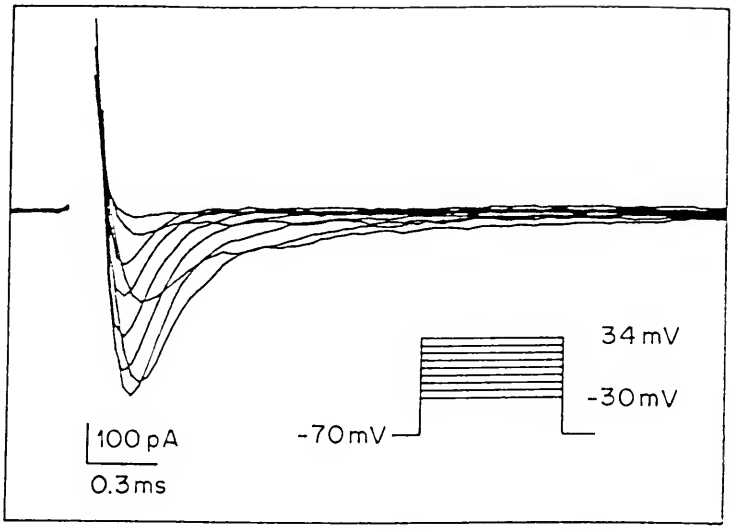
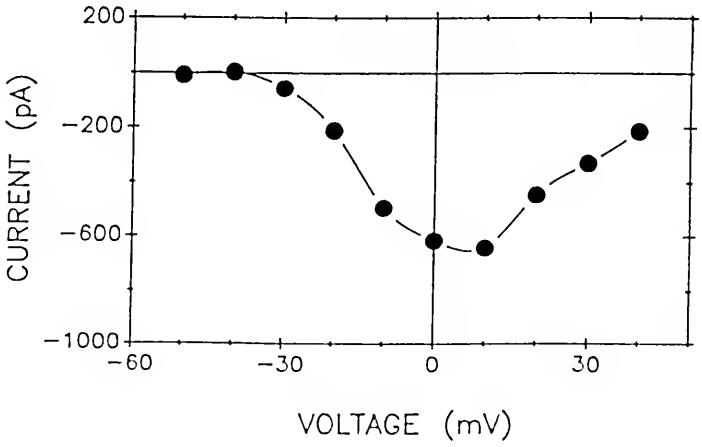

CURRENT (nA)
7
4
1
-2
-50
-30
-10
10
30
50
VOLTAGE (mV)

Fig. 2-2. The Na^+ current. Above: Transient inward currents elicited by depolarizing voltage steps (inset). Leak currents were not subtracted from these records. Below: The I-V relationship of this current. Activation began between -40 and -30 mV. The peak current occurred at 0 to 10 mV. Pipette (mM): 200 CsCl, 5 4-aminopyridine, 11 EGTA, 1 $CaCl_2$, 565 glucose, 10 HEPES, pH 7.4. Bath: saline, plus (mM); 4 $CoCl_2$, 4 $CdCl_2$, 10 TEA.


34 mV
-30 mV
-70 mV
100 pA
0.3 ms


200
-200
-600
-1000
CURRENT (pA)
-60
-30
0
30
VOLTAGE (mV)

linear regression of current amplitude at -6 mV versus cell capacitance (correlation coefficient = 0.30, N = 13 cells), but somata with a neurite, presumably an axon, had larger Na^+ currents, suggesting that axons contain higher densities of Na^+ channels. Steady state inactivation was half maximal at -48 mV (Fig. 2-3A; N = 7 cells). Inactivation was evoked by prepulses to a series of increasing potentials for 50 ms before the membrane potential was brought to 0 mV to elicit the Na^+ current. Recovery from inactivation followed a single exponential function with a mean time to half maximal activation of 1.7 ± 0.4 ms (N = 3 cells) at room temperature (Fig. 2-3B). Recovery from inactivation was measured using a two pulse protocol in which a series of paired pulses to 0 mV from a holding potential of -70 mV were separated by an increasing interval of time.

Ca^{++} current

A sustained inward current was isolated when Cs^+ replaced K^+ in the patch solution and 0.1 μM TTX was added to the bath (Fig. 2-4; N = 44 cells). This current, which washed out within minutes after breakthrough into the whole-cell configuration, was carried by either Ca^{++}, Ba^{++}, or Sr^{++} and was absent when either Co^{++} or Cd^{++}, or both, was added to the bath at a concentration of 4 to 10 mM (N = 65 cells). These observations identified this current as a Ca^{++} current. Like the Na^+ current, this current activated between -40 and -30 mV. When carried by Ca^{++} (N = 4 cells), the current decayed more rapidly (Fig. 2-5), suggesting that a Ca^{++}-dependent process is involved in the inactivation of this current. The washout of this current was not suppressed by 2 mM ATP and 0.2 mM GTP (N = 5 cells).

Fig. 2-3. Properties of inactivation of the Na^+ current. A. Steady state inactivation (h_∞) of the Na^+ current elicited by 50 ms prepulses to the potentials depicted on the abscissa. Shown are the means and standard deviations from four cells. Small standard deviations fall within circles marking the data points. B. Recovery from inactivation evoked by a paired pulse protocol. The plot denotes the amplitude of the Na^+ current evoked by the second pulse relative to the first versus the interval between the two pulses. The data points were fit by a single exponential curve with a time constant of 1.7 ms. Solutions as in Fig. 2-2.

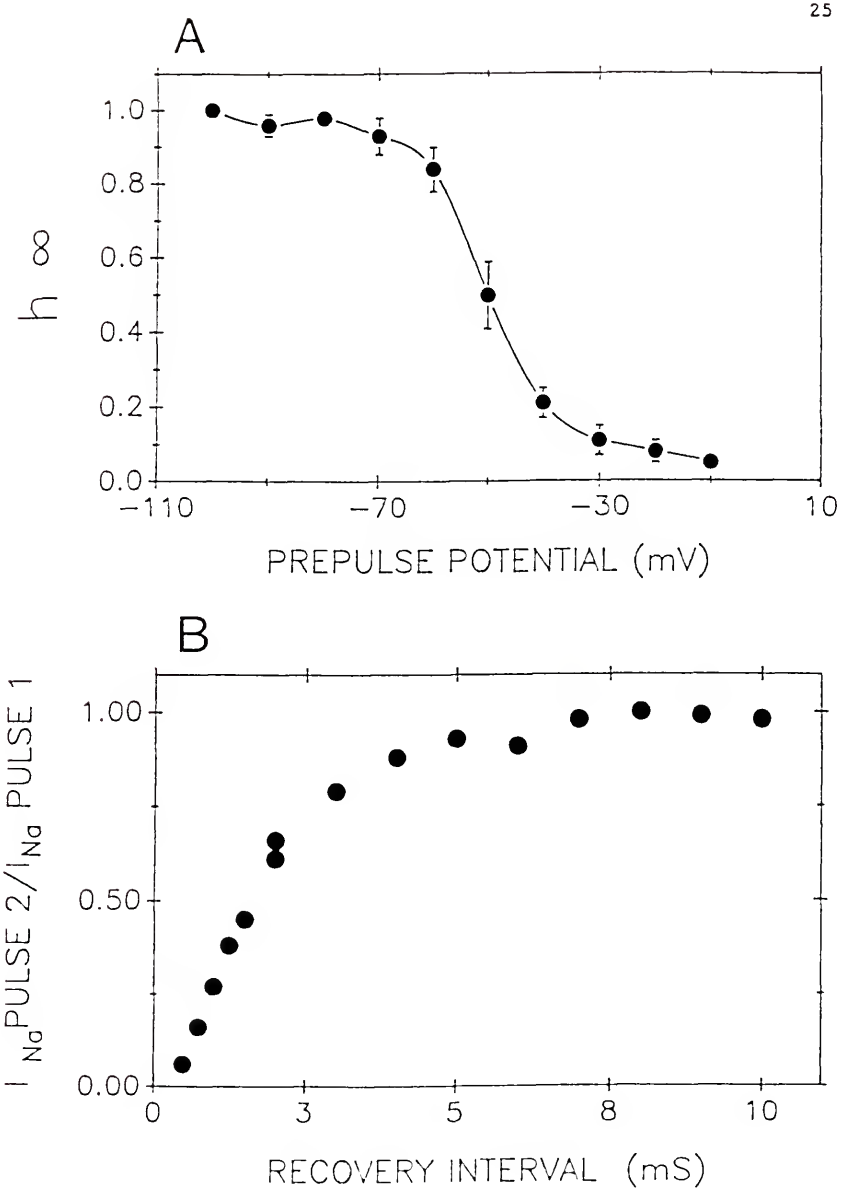
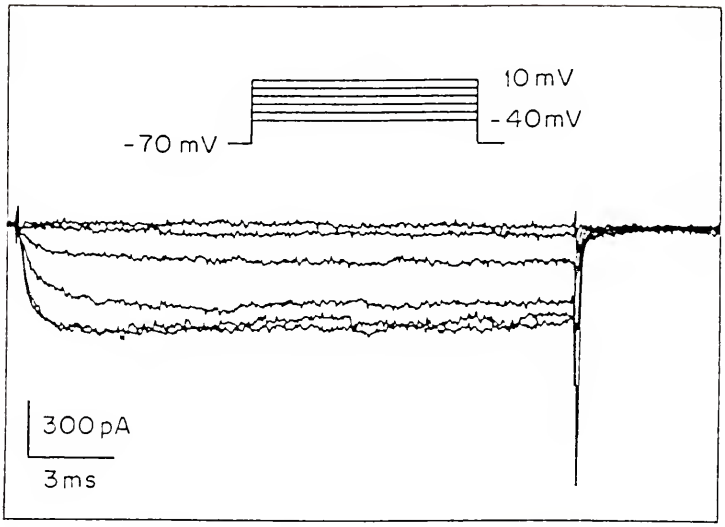

A
h ∞
1.0
0.8
0.6
0.4
0.2
0.0
−110
−70
−30
10
PREPULSE POTENTIAL (mV)
B
I Na PULSE 2/I Na PULSE 1
1.00
0.50
0.00
0
3
5
8
10
RECOVERY INTERVAL (mS)

Fig. 2-4. The Ca^{++} current. Above: Sustained inward currents carried by Sr^{++} elicited by depolarizing voltage steps (inset). Leak currents were subtracted from these records. Below: The I-V relationship of the current from the same cell. Activation began at about -40 mV. Peak current occurred at about -10 mV. Pipette (mM): 150 CsCl, 50 TEA, 5 4-aminopyridine, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 2 ATP, 0.2 GTP, 565 glucose, 10 HEPES, pH 7.4. Bath (mM): 480 NaCl, 13 KCl, 20 $SrCl_2$, 3 $MgCl_2$, 5 TEA, 20 HEPES, pH 7.4, and 0.1 μM TTX.


10 mV
-40mV
-70 mV
300 pA
3ms

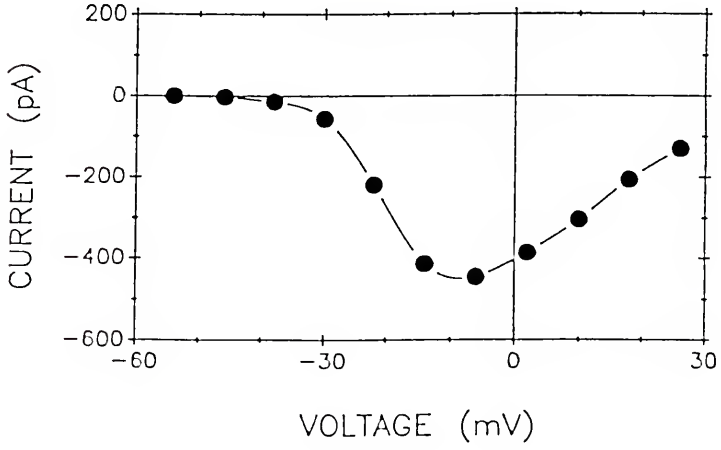
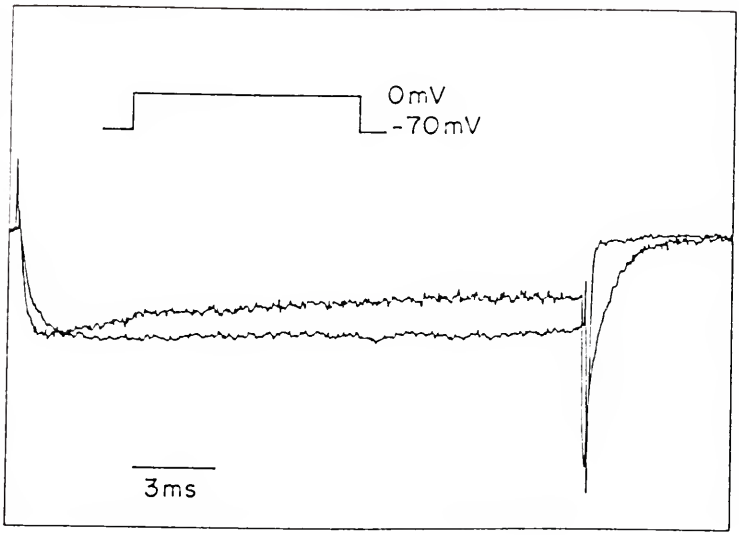

200
0
-200
-400
-600
-60
-30
0
30
CURRENT (pA)
VOLTAGE (mV)

Fig. 2-5. Currents through Ca^{++} channels evoked by depolarizing voltage steps to 0 mv (inset). Leak currents were subtracted from these records. Currents decayed more rapidly when carried by Ca^{++} (upper trace) than when carried by Sr^{++} (lower trace). Currents shown are from two different somata, normalized to the same amplitude and superimposed. Solutions as in Fig. 2-4 except that Ca^{++} replaced Sr^{++} in the saline bathing one cell.


0mV
-70mV
3ms

Ca^{++}-activated K^+ current

With 5 mM TEA and 0.1 μM TTX in the bath, a very large outward current was apparent (Fig. 2-6; N = 12 cells). Like the Ca^{++} current, this current disappeared within minutes after breakthrough into the whole-cell configuration. This washout was not suppressed by 1 mM dibutyryl cAMP (N = 3), which prevents washout of Ca^{++} currents in some neurons (Chad and Eckert, 1986). The current was absent if 4 mM Co^{++} or Cd^{++} was added to the bath (N = 55 cells) or if internal K^+ was replaced by Cs^+ or Na^+ (N = 64 cells). These observations identify this as a Ca^{++}-activated K^+ current. Applying 20 mM TEA internally (N = 2 cells) or 5 mM TEA externally (N = 6 cells) failed to block the current. The Ca^{++}-activated K^+ current decayed to varying degrees during voltage steps, apparently due to the inactivation of the Ca^{++} current, as the single channel underlying the Ca^{++}-activated K^+ current shows no inactivation (Chapter 3).

Delayed rectifier K^+ current

A delayed outward current was isolated by adding 0.1 μM TTX and 4 mM Co^{++} or Cd^{++}, or both, to the bath (Fig. 2-7; N = 55 cells). This current activated with a delay and inactivated slowly. The current was blocked by 5 mM external TEA (N = 6 cells) and was absent when internal K^+ was replaced by Cs^+ or Na^+ (N = 60 cells). Collectively, these features identify this current as a delayed rectifier type of K^+ current. Inactivation of this current was voltage dependent (Fig. 2-8). The inactivation elicited by 250 mS prepulses, which were too brief to achieve steady-state inactivation, was half-maximal at -41 mV (N = 4).

Fig. 2-6. The Ca^{++}-activated K^+ current. Above: The macroscopic currents evoked by depolarizing voltage steps (inset) immediately after breakthrough into the whole cell recording configuration. The Ca^{++} current providing the internal Ca^{++} for activation can be seen as the small inward current prior to the outward current. Leak currents were subtracted from these records. Below: The I-V relationship of the peak Ca^{++}-activated K+ current from the same cell immediately after breakthrough (circles) and 5 minutes later (triangles) illustrates the rapid washout of the Ca^{+} current. Pipette: normal patch solution plus 20 mM TEA. Bath: saline plus 4 mM TEA and 0.1 μM TTX.

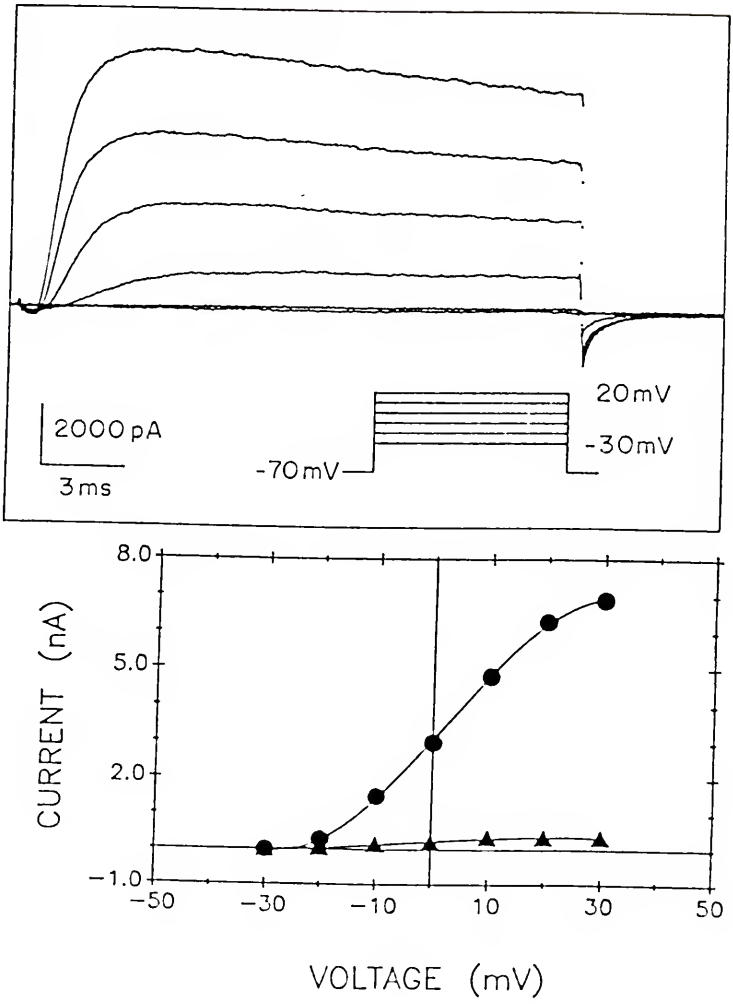
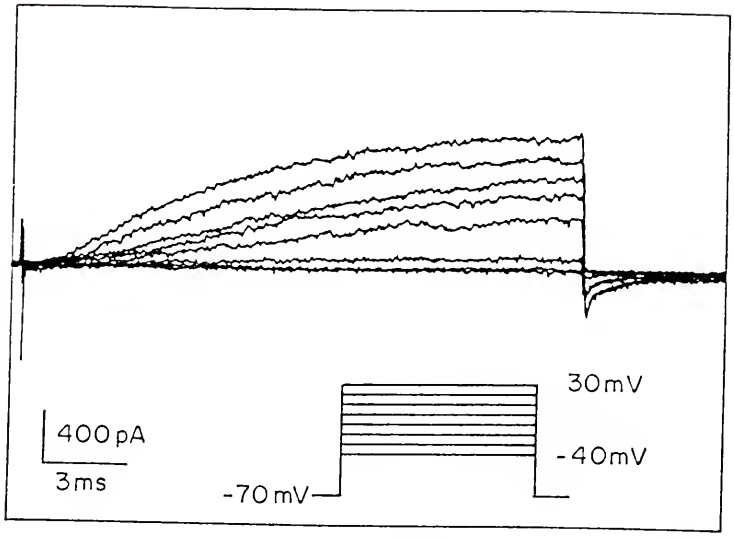

2000 pA
3 ms
-70 mV
20 mV
-30 mV
8.0
5.0
2.0
-1.0
CURRENT (nA)
-50
-30
-10
10
30
50
VOLTAGE (mV)

Fig. 2-7. The delayed rectifier K^+ current. Above: Macroscopic currents evoked by depolarizing voltage steps (inset) under conditions allowing isolation of the delayed rectifier current. Below: The I-V relationship of the peak current from the same cell. The current began to activate between -30 and -20 mV. Pipette: normal patch solution plus 2 mM ATP and 0.2 mM GTP. Bath: saline plus 4 mM $CoCl_2$, 4 mM $CdCl_2$ and 0.1 μM TTX.


30mV
-40mV
400pA
3ms
-70mV

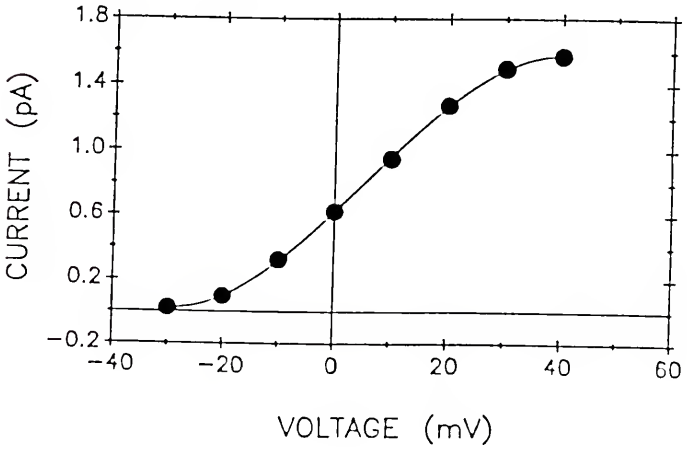
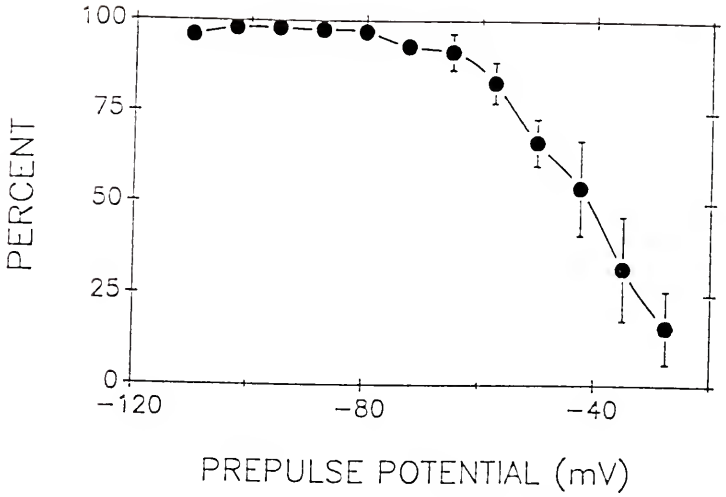

1.8
1.4
1.0
0.6
0.2
−0.2
CURRENT (pA)
−40
−20
0
20
40
60
VOLTAGE (mV)

Fig. 2-8. The voltage dependence of inactivation of the delayed rectifier current under conditions where steady-state inactivation had not been reached. The plot shows the percent of maximum current evoked at 40 mV versus the prepulse potential. The mean and standard deviation of 4 cells are plotted. Prior to stepping the holding potential to 40 mV to evoke the delayed rectifier current, 250 ms prepulses were applied in positive steps of 7.5 mV beginning at -110 mV. Between voltage step protocols the membrane was held at -70 mV. Solutions as in Fig. 2-6.


100
75
50
25
0
PERCENT
−120
−80
−40
PREPULSE POTENTIAL (mV)

This current was also modulated by at least one mechanism involving a GTP-binding protein. Perfusing somata with normal patch solution containing 20 mM NaF and 10 μM $AlCl_3$, a mixture that generates AlF_4^- which nonselectively activates GTP-binding proteins (Blackmore et al., 1985; Sternweis and Gilman, 1982), reduced the delayed rectifier over time (Fig. 2-9A vs. B). Analysis of variance showed that the reduction of the current in 5 somata perfused with this mixture was significantly greater than that of 7 somata perfused with normal patch solution ($p < 0.05$). Perfusing somata with normal patch solution containing 1 mM GTP-Γ-S, another GTP-binding protein activator, caused similar reductions in the current over time (Fig. 2-9C). Analysis of variance showed that the reduction of current in 5 somata perfused with GTP-Γ-S was significantly greater than that of the 7 somata perfused with normal patch solution ($p < 0.05$).

The delayed rectifier current was also blocked by forskolin. External application of 10 μM forskolin (N = 5 cells) caused a voltage-dependent increase in the rate of inactivation and decreased the amplitude of the delayed rectifier (Fig. 2-10). This particular effect of forskolin, however, has recently been shown to be a direct block of K^+ channels and not mediated by forskolin's ability to stimulate adenylate cyclase (Watanabe and Gola, 1987; Hoshi et al., 1988).

Discussion

Depolarizing receptor potentials evoked by odorants in lobster olfactory receptor cells can be larger than 40 mV and have plateau

Fig. 2-9. Effect of agents which activate GTP-binding proteins on the delayed rectifier current. A. The I-V relationships of the delayed rectifier current from an untreated cell showed little reduction over time. Plotted are current amplitudes immediately after breakthrough (circles), two minutes later (triangles), and 6 minutes later (squares). Solutions as in Fig. 2-6. B. The I-V relationships from a cell perfused with AlF_4^- shows reduction of the delayed rectifier current over time. Plotted are current amplitudes from immediately after membrane breakthrough (circles), 3 minutes later (triangles), and 6 minutes later (squares). Solutions as in Fig. 6 except that 20 mM NaF and 0.1 μM $AlCl_3$ were added to the patch solution. C. The I-V relationships from a cell perfused with GTP-Γ-S also shows reduction of the delayed rectifier current over time. Plotted are current amplitudes immediately after breakthrough (circles), two minutes later (triangles), and 5 minutes later (squares). Solutions as in Fig. 2-6, except that 0.5 mM GTP-Γ-S was added to the patch solution.

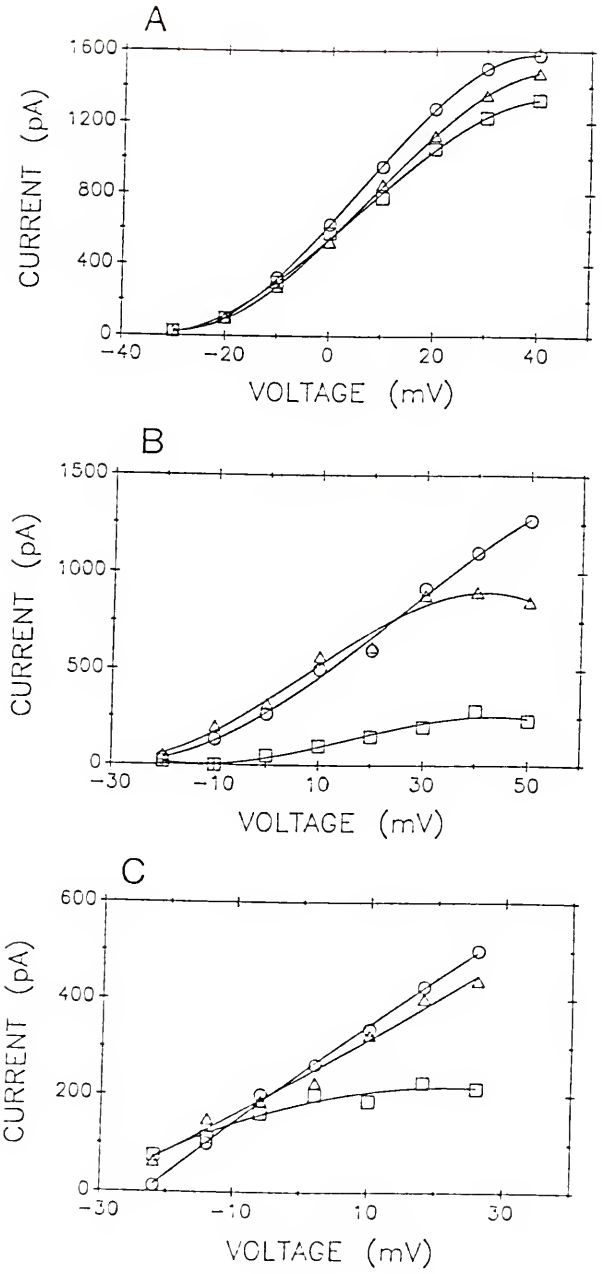
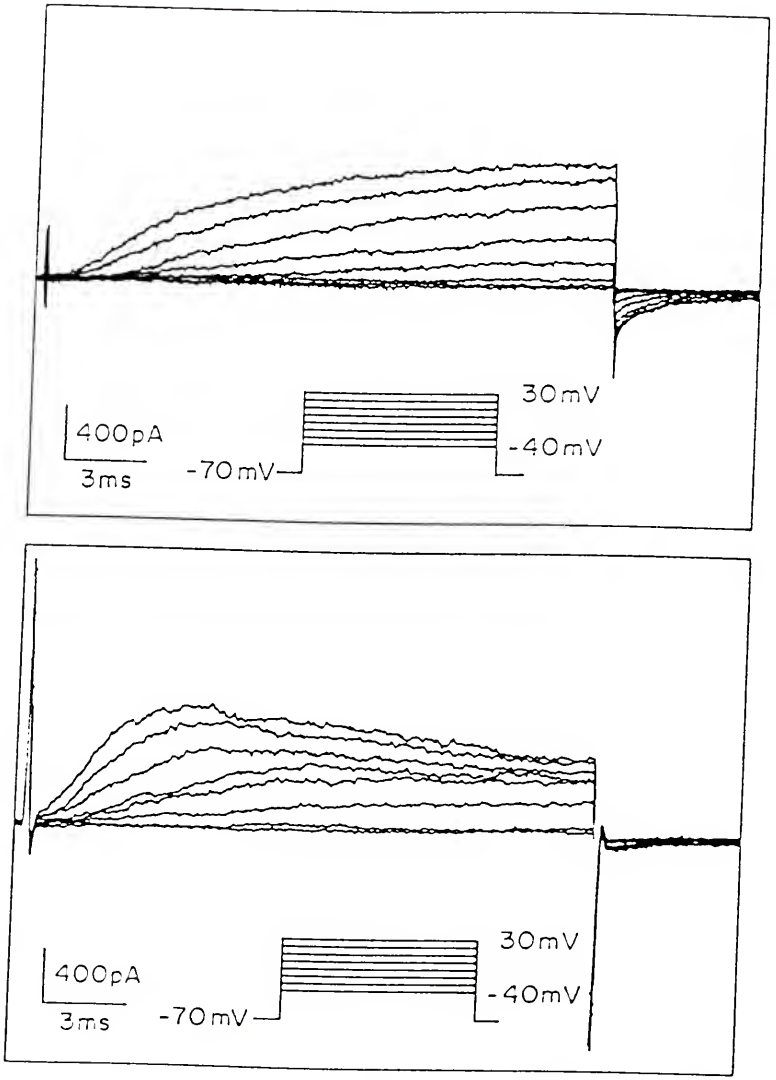

A
1600
1200
800
400
0
CURRENT (pA)
−40
−20
0
20
40
VOLTAGE (mV)
B
1500
1000
500
0
CURRENT (pA)
−30
−10
10
30
50
VOLTAGE (mV)
C
600
400
200
0
CURRENT (pA)
−30
−10
10
30
VOLTAGE (mV)

Fig. 2-10. The effect of forskolin upon the delayed rectifier current. Above: An untreated cell showed a normal family of delayed rectifier currents. Below: In the presence of 10 μM forskolin, the delayed rectifier current in a neighboring cell showed voltage-dependent inactivation. Solutions as in Fig. 2-6 except that forskolin was added to the bath between recording from the two cells.


400pA
3ms
-70mV
30mV
-40mV
400pA
3ms
-70mV
30mV
-40mV

potentials lasting at least a few hundred milliseconds (Anderson and Ache, 1985; Schmiedel-Jakob et. al., 1989). My results support the hypothesis that these receptor potentials spread passively from the dendrite and generate Na^+ action potentials in the axon. I found no evidence of a voltage-dependent inward current active at potentials more negative than the Na^+ current, or of an ongoing outward current decreased by potentials between rest and threshold, either of which could increase the effective space constant of the cell (Yoshii et al., 1988). This suggests that the spread of receptor potentials, at least through the soma, is passive. Unless the dendrite has voltage-dependent currents not found in the soma, one must assume that the passive membrane properties of the cell must account for the spread of current. My observation that the size of the Na^+ current correlated not with the size of the soma, but with the presence of a remnant of neurite, suggests that most Na^+ channels are located on the neurite. Assuming that the neurites were axons would be consistent with intracellular recordings from intact cells indicating that the axon is the site of generation of action potentials in lobster olfactory receptor cells (Schmiedel-Jakob et al., 1989). Furthermore, the relatively negative potentials at which the Na^+ current inactivates suggests that any Na^+ channels present in the soma and dendrite would rapidly inactivate during a receptor potential. Trotier (1986) similarly concluded that the axon contains most of the Na^+ current based on its infrequent occurrence in isolated salamander olfactory receptor cells.

Because 1 mM external TEA broadens action potentials and reduces outward rectification in these cells (Schmiedel-Jakob et al., 1989), the delayed rectifier K^+ current may participate in both repolarization of the action potential and in the outward rectification of the cell. The Ca^{++}-activated K^+ current could also be involved in both these functions as well because it activates almost as rapidly as a similar current involved in repolarization of the action potential in bullfrog sympathetic neurons (MacDermott and Weight, 1982) and because it takes more than 100 ms to decay. Predictions about functions of the Ca^{++}-activated K^+ current have been difficult to test, however, due to the rapid washout of the Ca^{++} currents in the whole-cell recording configuration.

Outward rectification produced by the K^+ currents may act to expand the range of odor-evoked inward currents which can be encoded into changes in spike frequency. Given that the interval of potential from threshold to saturation of spike frequency is fixed in any one cell, from Ohm's law, the amount of inward current capable of causing potentials that fall within this interval would increase as the K^+ currents decrease the membrane resistance. That this occurs is demonstrated by TEA block of the delayed rectifier current: the injection of 100 pA of current into an intact lobster olfactory receptor cell caused a potential change of 23 mV before, and 47 mV after, TEA application (Schmiedel-Jakob et al., 1989).

Unlike amphibian olfactory receptor cells (Firestein and Werblin, 1987; Trotier, 1986; Suzuki, 1987), the somata of lobster olfactory receptor cells lack an A current or other transient

outward current. In many neurons, an A current appears to be necessary to allow repetitive spiking over a wide range of frequencies (Connor, 1976). How the lobster olfactory receptor cell accomplishes repetitive spiking over a wide range of frequencies (Schmitt and Ache, 1979) in the absence of an A current is unclear. Perhaps one or both of the K^+ currents subserves this function by slowing the return to threshold following a spike. A Ca^{++}-activated K^+ current appears to permit repetitive spiking in frog motoneurons (Barrett and Barrett, 1976). Mouse olfactory receptor cells also appear to lack the K^+ channel underlying the A current (Maue and Dionne, 1987), suggesting that multiple mechanisms exist for controlling spike frequency in olfactory receptor cells.

The depression of the delayed rectifier current by forskolin and by agents which activate GTP-binding proteins illustrates a caveat for the somatic injection of agents intended to act upon transduction mechanisms in neuritic processes. Care must be taken to ensure that such agents have specific actions which occur only in the desired location within the cell.

In conclusion, lobster olfactory receptor cells appear to perform the same functions as their amphibian counterparts, but with a slightly smaller number of voltage-dependent currents. At present, these results suggest that the Na^+ current, located mostly in the axon, underlies action potentials in lobster olfactory receptor cells, while the Ca^{++} and K^+ currents shape the plateau of the receptor potential, repolarize the membrane, and control repetitive spiking. Recording of odor-evoked responses from intact

cells while selectively blocking each of the cell's ionic currents, would test the proposed physiological roles of these four currents.

CHAPTER 3

SOMATIC ION CHANNELS

Introduction

Olfactory receptor cells are primary sensory neurons that transduce chemosensory information into graded electrical signals and generate action potentials that propagate to the CNS. The description of the ion channels which underlie these electrical responses is a step toward understanding olfactory receptor cell function. Because these are bipolar neurons, the ion channels of the soma interact with odor-evoked membrane potential changes spreading from transduction sites in the dendrites (reviewed by Getchell and Getchell, 1987) to the axon where action potentials are presumably generated (Getchell, 1973; Masukawa et al., 1985; Trotier, 1986; Schmiedel-Jakob et al., 1989).

To date little information exists about the somatic ion channels of olfactory receptor cells. Mouse olfactory receptor cells have two Ca^{++}-activated K^+ channels, an inward rectifier, a delayed rectifier, a Cl^- channel, and a Ca^{++} channel (Maue and Dionne, 1987). K^+ channels have also been observed in salamander olfactory receptor cells (Trotier, 1986). Because the morphology of lobster and vertebrate olfactory receptor cells is similar (Grünert and Ache, 1988), a comparison of ion channel properties may indicate

electrical properties which are common to olfactory receptor cells in general, and identify potential roles of the channels in the function of lobster olfactory receptor cells.

In this chapter I characterize four ion channels found on the soma of the lobster olfactory receptor cell and correlate their properties with functions of the receptor cell. These channels are a steady state Cl^- channel, a Ca^{++}-activated K^+ channel, a voltage-activated K^+ channel, and a weakly voltage-dependent cation channel.

Methods

Preparation of receptor cells

Specimens of the spiny lobster, P. argus, were collected in the Florida Keys and maintained in running seawater for up to three months. Olfactory receptor cells were prepared for patch-clamp recording by treating hemicylinders of cuticle containing the cells with enzymes as described in Chapter 2. Hemicylinders, or isolated somata, were placed in a 35 mm culture dish for patch clamp recording. Cells were viewed at 200X or 300X under brightfield or modulation contrast optics.

Patch clamp recordings

Patch clamp pipettes were made from borosilicate glass pipettes (H15/10/0181, Jencons Scientific Ltd., Leighton Buzzard, England) pulled and fire-polished to tip diameters of less than 1 μm (bubble numbers of 3 to 4; Mittman et al., 1987). Sylgard 184 (Dow Corning, Midland, MI) was applied to the electrode neck to reduce capacitance when attempting to record channels rapidly activated by step changes in holding potential. Single channel currents were amplified with a

patch clamp amplifier and a 10 gigaohm head stage (Dagan Instruments, Minneapolis, MN). Records were low-pass filtered at 10 kHz for storage on video tape (Bezanilla, 1985) and at 0.5 to 5 kHz for analysis, using an eight pole low-pass Bessel filter (Frequency Devices, Inc., Haverhill, MA). An IBM XT with an A/D, D/A converter and accompanying software (pClamp, Axon Instruments, Burlingame, CA) was used both to apply voltage step protocols and to digitize and analyze single channel records. Kinetic analysis was performed only on records from patches containing one active channel. Open and closed duration distributions were compiled as histograms and exponential probability density functions were fitted to them by a chi-square minimization method. For cell-attached patches, voltages are reported as the voltage applied across the membrane, without reference to the contribution of the cell's membrane potential (not measured), following the convention of inside as negative. Values are reported as means $\pm$ standard deviations.

Solutions

The compositions of patch solutions and salines are listed in Table 3-1. HEPES and EGTA were obtained from Research Organics, Inc. (Cleveland, OH) and inorganic salts from Fisher Scientific (Fair Lawn, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium concentrations in EGTA solutions were calculated according to Hagiwara (1983). Solutions were buffered to pH 7.4 with HEPES.

Solution changes were made by moving inside-out patches through a slot cut into 2 concentric Delrin rings, one of which was fixed in

TABLE 3-1. Composition of solutions used, in mM

	Saline	Low divalent saline	Ca^{++} saline	Ba^{++} saline	KCl saline	NaCl saline	Low Cl saline	Na^{+} patch
NaCl	460	480	420	420		480	350	190
KCl	13	13			480			
$CaCl_2$	13	.01	60		.01	.01	.01	1
$MgCl_2$	10							
$BaCl_2$				60				
EGTA								11
HEPES*	10	20	10	10	20	20	10	20
glucose		1.7			1.7	1.7		598
NaOAc							130	

* Either NaOH or KOH was used to adjust the pH of solutions in order to allow preparation of pure Na^{+} and K^{+} salines.

the bath. The outer ring was then turned about the fixed inner ring to isolate a 300 µl chamber from the remainder of the bath. A push-pull syringe system allowed changing the solution in the chamber independently of that in the remainder of the bath. Alternatively, the patch was moved into a flow from a glass pipette (100 µm tip diameter) connected by a switching valve to six solution reservoirs.

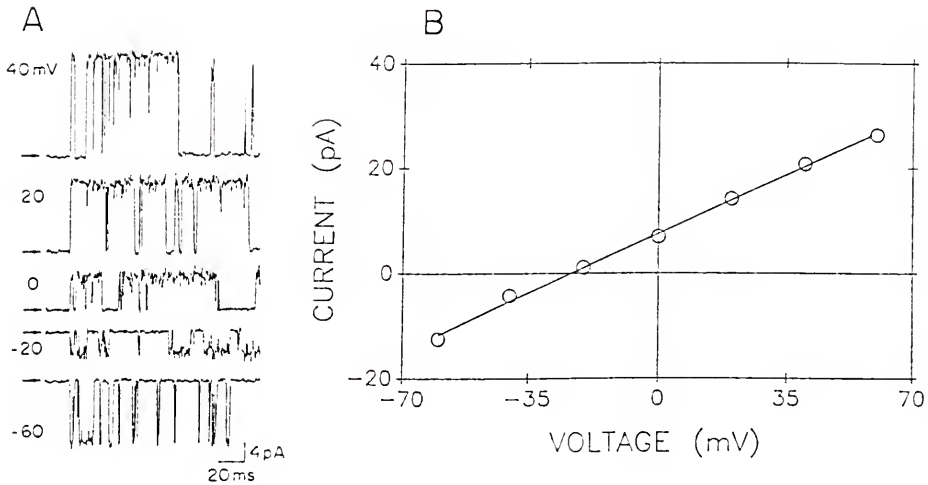
Results

A voltage-dependent cation channel

Conductance and effects of monovalent ions. The most frequently observed channel in inside-out patches was a nonselective cation channel. This channel was never clearly observed in cell-attached patches. It had a linear I-V relationship with low divalent saline on its cytoplasmic face and Na^+ patch solution on its extracellular face (Fig. 3-1A, B). The mean slope conductance was 320 ± 21 pS (N = 19). Replacing Na^+ with K^+ in the solution on the channel's extracellular face or with Cs^+ on its cytoplasmic face caused the reversal potential to shift to values intermediate between the Nernst potentials of Na^+ and K^+ or Cs^+. Permeability ratios for K^+ relative to Na^+ were 0.66 and 0.74 for the two channels tested, and for Cs^+ relative to Na^+ were 0.33 and 0.35 for two other channels. Substituting glutamate or gluconate for Cl^- on the cytoplasmic face (N = 3) failed to shift the reversal potential.

Replacing the 480 mM Na^+ in the intracellular solution with Cs^+ or K^+ caused a gradual reduction in the frequency of channel

Fig. 3-1. A nonselective cation channel in an inside-out patch bathed in Na^+ patch solution and low divalent saline. A. Representative records of the channel under conditions where Na^+ was the major charge carrier. Arrows indicate the current level of the closed state. Corner frequency (f_c) = 2 kHz. B. A plot of the I-V of the channel depicted in A. The slope conductance was 323 pS.


A
40mV
20
0
-20
-60
4pA
20ms
B
CURRENT (pA)
40
20
0
-20
-70
-35
0
35
70
VOLTAGE (mV)

openings and total lack of activity within a few minutes. Reducing the Na^+ concentration to 20, 50, 100, or 200 mM by K^+ or Cs^+ substitution caused the same effect. Adding back the 480 mM Na^+ caused recovery of channel activity in only 3 of 8 patches. The temporal correlation between these solution changes and changes in channel activity was poor, suggesting that the effect was not a simple Na^+ activation of gating.

Single channel kinetics. Subsequent measurements of the channel's properties were performed on inside-out patches with an intracellular solution containing 460 or 480 mM Na^+. With high intracellular Na^+, the cation channel rarely became inactive, even in patches which lasted longer than one hour. The probability of being in the open state (P_o) was increased by depolarization (Fig. 3-2A). Voltage dependence was analyzed by fitting these data to a linearized form of the Boltzmann equation (Fig. 3-2B). Apparent gating charges, calculated from the slopes of these lines, had a mean value of 1.11 ± .06 (N = 5). The average half-maximal activation voltage was -37 ± 7 mV. The increase in P_o caused by depolarization was due to both increased mean open times and decreased mean closed times (Fig. 3-3). Most open dwell time distributions were fit best by a single exponential (Fig. 3-4A) whereas closed dwell time distributions usually were better fit by a double exponential (Fig. 3-4B).

None of the properties of the cation channel were affected by perfusing the intracellular side of the patch with salines containing 10^{-10} M to 10^{-4} M Ca^{++}, nor were they affected by 10 μM

Fig. 3-2. The voltage dependency of the probability of the nonselective cation channel being in the open state (P_o). A. Plot of the relationship of P_o and voltage for one channel. The curve was fit to the data (points) by nonlinear regression. B. Plot of the Boltzmann linearization of the data from A. The line was fit to the data by linear regression.

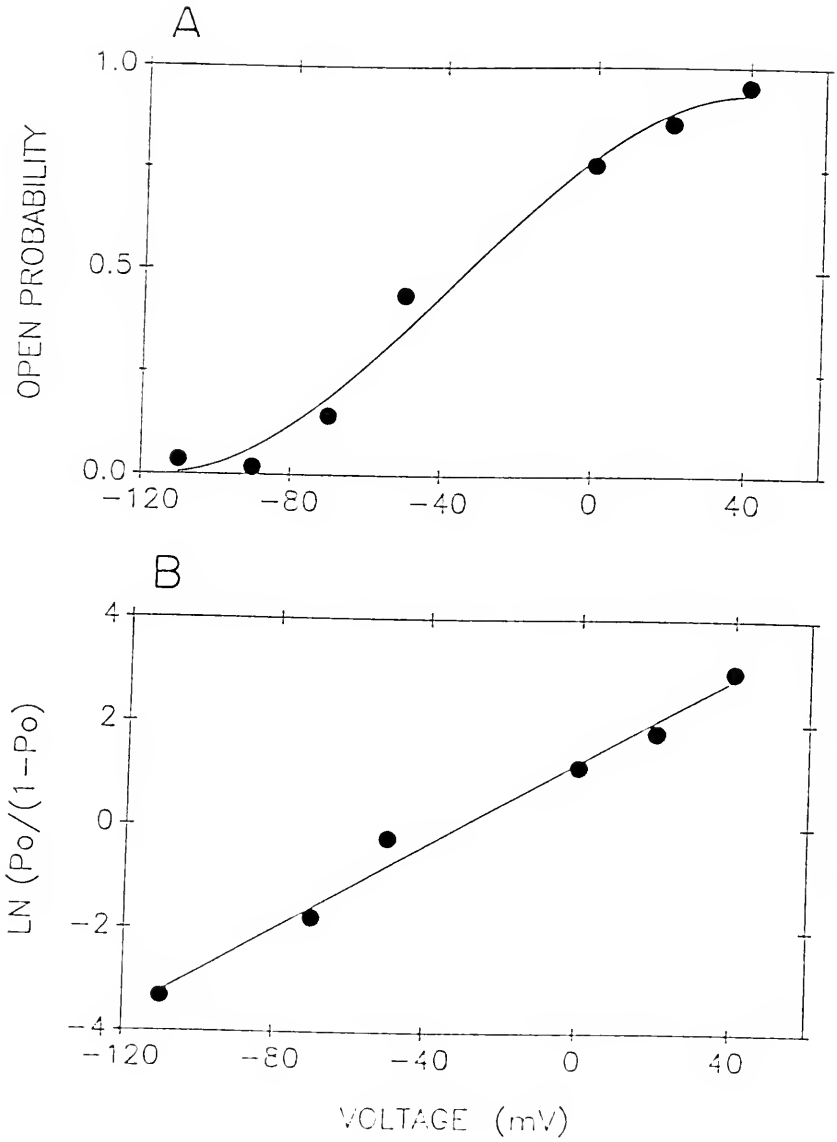

A
1.0
0.5
0.0
OPEN PROBABILITY
−120
−80
−40
0
40
B
4
2
0
−2
−4
LN (Po/(1−Po))
−120
−80
−40
0
40
VOLTAGE (mV)

Fig. 3-3. Plot of voltage dependence of the mean open (closed circles) and closed times (open circles) of a nonselective cation channel. Depolarization increased the P_o by increasing the mean open time and decreasing the mean closed time. The curves were fit to the data by nonlinear regression.

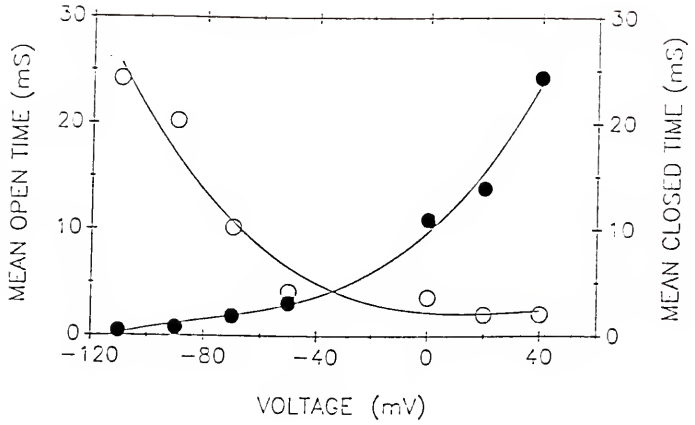
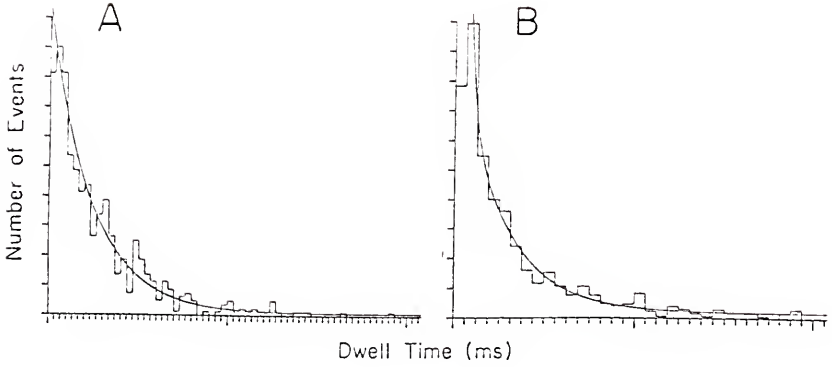

MEAN OPEN TIME (mS)
MEAN CLOSED TIME (mS)
VOLTAGE (mV)
30
20
10
0
−120
−80
−40
0
40

Fig. 3-4. Representative plots of the distributions of the open and closed dwell times of a nonselective cation channel. Dwell times were plotted as histograms and then exponential curves were fit to the binned data by a chi-square minimization method. A. The distribution of a record of 602 open dwell times at 20 mV fit by a single exponential with a time constant of 13.9 ms. B. The distribution of the closed dwell times from the same recording at 20 mV fit by a double exponential curve with time constants of 0.2 ms and 2.0 ms.

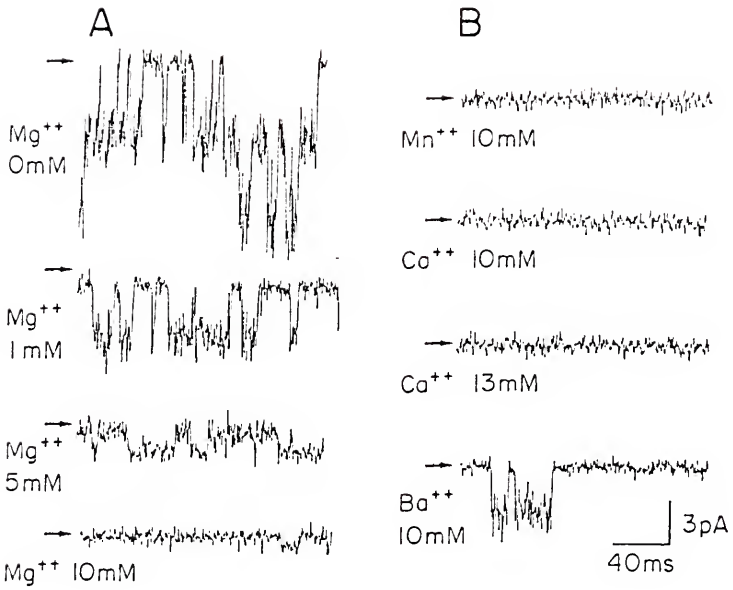

A
B
Number of Events
Dwell Time (ms)

cGMP (N = 10) or 10 μM cAMP (N = 8) applied to the same side. External TTX (N = 1) and mouth suction or pressure applied to the pipette also had no discernible effect. A potent odor mixture (a filtered, saline extract of TetraMarin, TetraWerke, Melle, FRG) applied in the patch pipette failed to cause the appearance of this channel (or any other channel) in cell-attached patches or to alter the properties of the cation channel in inside-out patches (N = 33).

Block by tetraethylammonium and divalent cations. Perfusion of the intracellular side of the channel with salines containing 20 or 50 mM TEA caused a voltage dependent channel block characterized by rapid flickering between the open and closed current levels (data not shown). Block by TEA was not pursued further. Block by internal divalent cations, however, provided clues about the pore morphology and kinetic properties of this channel. Ba^{++}, Ca^{++}, Mn^{++}, Co^{++}, and Mg^{++}, at concentrations from 1 to 13 mM, all reduced the open channel amplitude in a manner characteristic of very fast blocking kinetics (Fig. 3-5A, B). The block by Mg^{++}, the only divalent cation likely to occur at millimolar intracellular concentrations, was investigated in detail.

In fast block, Mg^{++} decreased the apparent open channel amplitude in a concentration-dependent manner (Fig. 3-5A). The dissociation constant of Mg^{++} for the binding site, or sites, responsible for fast block was obtained by analyzing the blocker titration curve of Mg^{++} (Fig. 3-6A) in which the ratio of the blocked to the unblocked channel amplitude (i_B/i) is the fraction of time a channel is not occupied by the blocker (Moczydlowski, 1986).

Fig. 3-5. Representative records showing the blocking effect of divalent cations applied to the intracellular side of inside-out patches containing a nonselective cation channel. A. The concentration dependence of block by Mg^{++}. All records obtained from the same patch containing 3 cation channels. B. 10 mM or more Mn^{++}, Co^{++}, and Ca^{++} appeared to reduce the open channel amplitude below the limit of resolution. 10 mM Ba^{++} blocked the channel less effectively than the other ions. These records were from several different patches containing a single cation channel. All records were obtained at -25 mV in symmetrical 480 mM NaCl and low pass filtered at a corner frequency of 1.5 kHz. Arrows indicate the current level of the closed state.


A
B
Mg++ 0mM
Mg++ 1mM
Mg++ 5mM
Mg++ 10mM
Mn++ 10mM
Co++ 10mM
Ca++ 13mM
Ba++ 10mM
3pA
40ms

At binding equilibrium between n blocker molecules and the open channel (1), the equations describing the blocker titration curve (2) and its Hill plot (3) are

$$nB + O \underset{}{\overset{K_B}{\longleftrightarrow}} nB \cdot O \qquad (1)$$

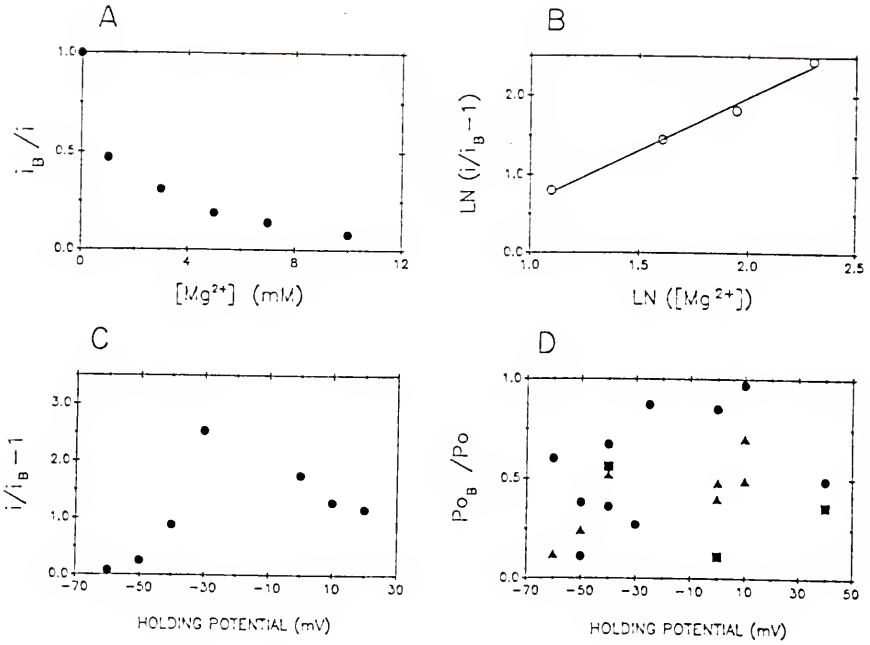
$$i_B/i = (1 + [B]^n/K_B)^{-1} \qquad (2)$$

$$\ln(i/i_B - 1) = n \ln[B] - \ln K_B \qquad (3)$$

where [B] is the concentration of blocker, n is the Hill coefficient, and K_B is the dissociation constant of the blocker. Hill coefficients and dissociation constants obtained from two patches exposed to 1 to 10 mM Mg^{++} were 1.34 and 2.0 mM for one patch and 1.61 and 6.4 mM for the other (Fig. 3-6B). Hill coefficients of greater than one indicate that more than one ion is involved in fast block.

Fast block by Mg^{++} was also voltage-dependent (N = 5), reaching a maximum between -30 and -10 mV (Fig. 3-6C), possibly corresponding to the Na^+ reversal potential (E_{Na}) of -23 mV. This observation suggested that Mg^{++} interacted with Na^+ in the permeation pathway and that Mg^{++} might even be slightly permeable. The voltage dependence of the fast block was analyzed according to the Woodhull (1973) model which assumes that voltage dependence arises because the binding site for a charged blocking particle lies within the membrane's electric field, for example, in the channel pore. The data from membrane potentials below E_{Na} were linearized and estimates of K_B and of $z\delta$, the proportion of the membrane's electric field sensed by a charged blocking particle at its binding site,

Fig. 3-6. Analysis of the Mg^{++} block of the nonselective cation channel. A. A representative plot of the blocker titration curve of one channel; the blocked single channel current normalized to the unblocked current as a function of the concentration of Mg^{++} at 0 mV. B. Plot of the fit of the Hill equation to the data in A. A linear regression of these data yielded a Hill coefficient of 1.34 and a dissociation constant of 2.0 mM. C. Plot of the voltage dependence of block of 3 mM Mg^{++}; block is relieved to differing extents by hyperpolarization and depolarization. D. A plot of voltage versus the normalized reduction of the P_o caused by intracellular Mg^{++} shows that the effect of Mg^{++} on channel gating is relatively independent of voltage. 1 mM Mg^{++} (circles), 3 mM Mg^{++} (squares), 5 mM Mg^{++} (triangles).


A
B
C
D
i_B/i
$[Mg^{2+}]$ (mM)
LN $(i/i_B - 1)$
LN $([Mg^{2+}])$
$i/i_B - 1$
HOLDING POTENTIAL (mV)
Po_B/Po
HOLDING POTENTIAL (mV)

were obtained from the following equation (Moczydlowski et al., 1986):

$$\ln[(i/i_B) - 1] = z\delta FV/RT + \ln([B]/K_B) \quad (4)$$

Estimates of K_B averaged 39 ± 16 μM Mg^{++} (N = 5). Estimates of $z\delta$ averaged 2.6 ± 0.2 (N = 5), indicative of multiple blocker binding sites.

In addition to fast block, Mg^{++} also caused a concentration-dependent reduction in P_o, termed slow block, that was voltage independent (Fig. 3-6D). The difference in voltage dependency between fast and slow block suggests that distinct binding sites, or sets of binding sites, mediate the two types of block. Slow block was not limited to Mg^{++}, as 10 mM Ba^{++} applied internally reduced P_o from 0.84 to 0.25 at -20 mV.

Permeability to Ca^{++} and Ba^{++}. The finding that holding potentials more positive than E_{Na} relieved the block by Mg^{++} raised the possibility that divalent cations could permeate this channel. To test this possibility, the internal faces of inside-out patches containing cation channels were perfused with 60 mM divalent cations. This manipulation, however, eliminated all channel activity, possibly due to the slow block by divalent cations described above. Subsequently, channel activity which may correspond to the cation channel was easily found in inside-out patches from cells bathed in low divalent saline when either Ca^{++} or Ba^{++} saline was applied the extracellular side of inside-out patches. Under these conditions, inward current events were observed at potentials above E_{Na} (-3 mV) and E_{Cl} (-11 mV) where they

could only be carried by the divalent cation (Fig. 3-7A, B; Chesnoy-Marchais, 1985). The properties of these divalent permeable channels; no inactivation, weak voltage dependence, and presence in inside-out, but not cell-attached patches, correlate with those of the cation channel. The conductance of the channel in Ba^{++} saline was 80 pS, while values of 43 and 44 pS were obtained in Ca^{++} saline (Fig. 3-7C). If these channels were the same cation channel found in solutions with low divalent cation concentrations, the P_o in Ba^{++} saline was much reduced (e.g., P_o = 0.39 at -5 mV versus > 0.9, cf Fig. 3-2). The effect of Ca^{++} saline was even more dramatic in that the P_o increased to 0.1 at -10 mV, but failed to increase further with stronger depolarization.

A cell-attached cation conductance. The nonselective cation channel was never clearly apparent in cell-attached patches, even in those which demonstrated cation channel activity after excision to the inside-out configuration. With NaCl or KCl solutions containing micromolar divalent cations in the patch pipette, however, large inward currents whose kinetics were too rapid to permit resolution of the current amplitude occurred in cell-attached patches on cells bathed in low divalent saline (Fig. 3-8). This cell-attached channel activity was not observed with millimolar concentrations of divalent cations in the pipette, and only rarely occurred in cells bathed in salines containing millimolar concentrations of divalent cations. Depolarization decreased the apparent amplitude and increased the frequency of these current transients. When excised into the inside-out configuration, 65% of these patches contained

Fig. 3-7. Inward current events carried primarily by Ba^{++} and Ca^{++} through putative nonselective cation channels in inside-out patches. A. Representative records showing inward current events in Ca^{++} saline. The patch contained two channels, indicated by simultaneous open events summing to twice the single channel current amplitude (not shown). Arrows indicate the current level of the closed state. f_c = 1 kHz. B. Representative records showing inward current events in Ba^{++} saline. The patch contained three channels. f_c = 1 kHz. C. Plot of the current-voltage relationships of the data represented in A. and B. in external Ca^{++} saline (closed circles) and in Ba^{++} saline (open circles). Note the inward rectification. Curves were fit to the data by nonlinear regression.

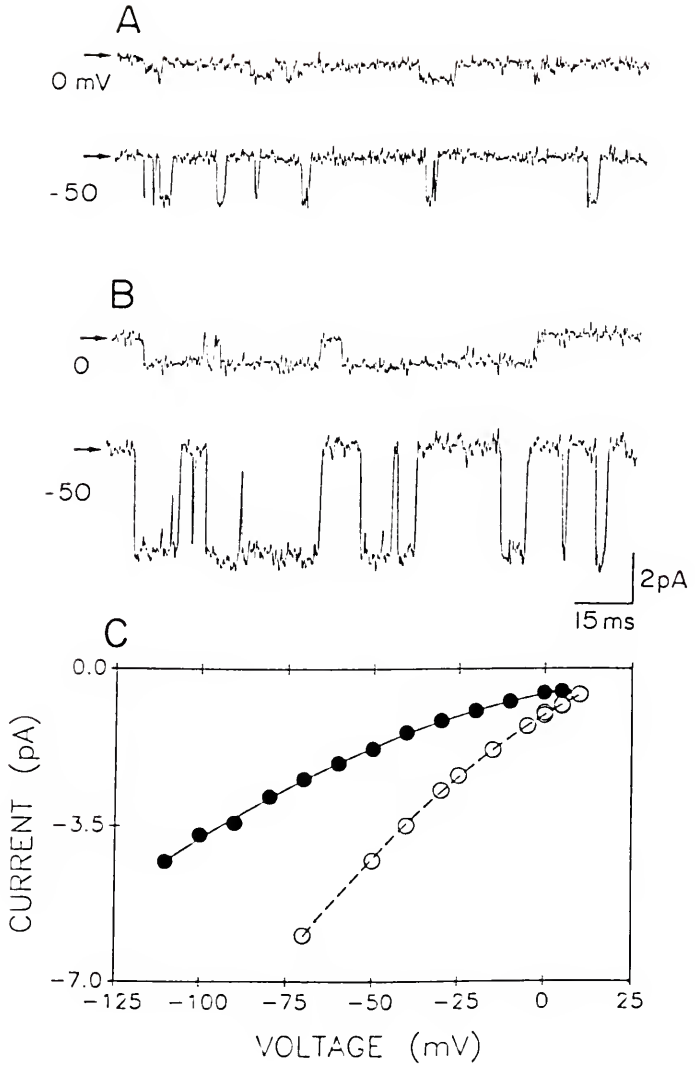
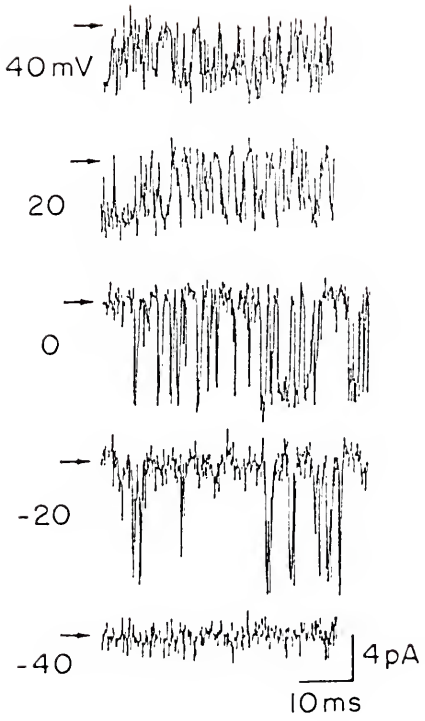

A
0 mV
-50
B
0
-50
2pA
15 ms
C
0.0
-3.5
-7.0
CURRENT (pA)
−125
−100
−75
−50
−25
0
25
VOLTAGE (mV)

Fig. 3-8. Representative records of a putative nonselective cation channel in a cell-attached patch. Note the rapid transitions between the open and closed states of the channel. This was the record with the slowest kinetics observed. The pipette contained NaCl saline. Arrows indicate the current level of the closed state. f_c = 2 kHz.


40 mV
20
0
-20
-40
4 pA
10 ms

one or more of the familiar nonselective cation channels, but no other channel activity. Less than 40% of the silent cell-attached patches showed cation channel activity after being excised. Contingency table analysis of these proportions showed a significant interaction of cell-attached and inside-out cation channel activity (chi square = 4.1, $p < 0.05$, 1 df, N = 119). These observations allow that the vastly different types of kinetic activity observed could have arisen from a single species of cation channel responding to the greatly different biochemical environments imposed by cell-attached and inside-out recording configurations.

A Ca^{++}-activated K^+ channel

A large-conductance, Ca^{++}-activated K^+ channel was observed in both cell-attached and inside-out patches. In symmetrical 480 mM KCl, its I-V relationship (Fig. 3-9A) had a slope conductance of 215 ± 33 pS (N = 5). The average permeability ratio of K^+ to Na^+, calculated from inside-out patches with Na^+ patch solution on the extracellular side of the patch and KCl saline on the intracellular side, was 3.9 ± 0.9 (N = 9). Increasing the Ca^{++} concentration bathing the internal face of this channel from 10^{-7} M to 10^{-6} M or 10^{-5} M increased the frequency of opening (Fig. 3-10). At a constant internal Ca^{++} concentration, depolarization also increased the P_o by increasing the mean open time, with a weak tendency to decrease the mean closed time as well (Fig. 3-9B). Neither 20 mM TEA, 5 mM 4-aminopyridine, nor 10 mM Cs^+ in KCl saline blocked this channel when applied to its intracellular side.

Fig. 3-9. Plots of the I-V relationship and kinetic properties of a Ca^{++}-activated potassium channel. A. The current-voltage relationship of a single channel in an inside-out patch in symmetrical 480 mM KCl. The slope conductance was 197 pS. B. The mean open (open circles) and closed (closed circles) times versus voltage. Na^{+} saline containing 10^{-5} M Ca^{++} has been substituted as the intracellular solution. The curves were fit to the data by nonlinear regression.

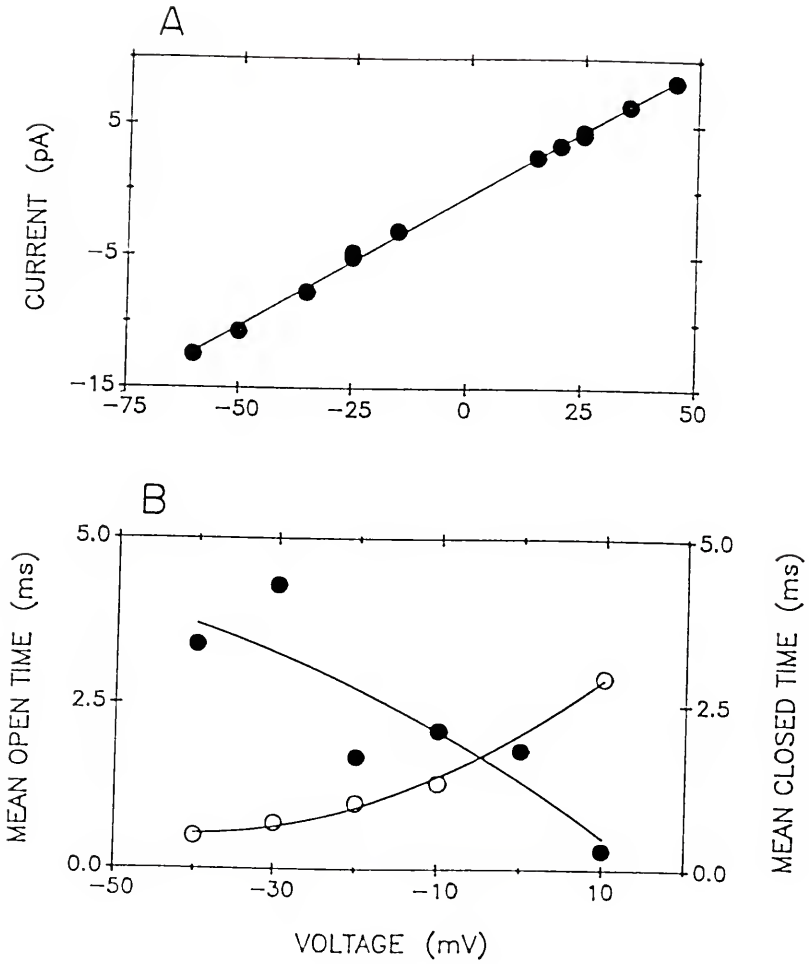
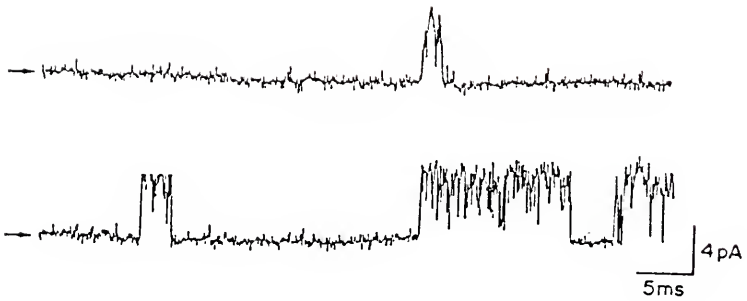

A
CURRENT (pA)
5
−5
−15
−75
−50
−25
0
25
50
B
MEAN OPEN TIME (ms)
5.0
2.5
0.0
−50
−30
−10
10
MEAN CLOSED TIME (ms)
VOLTAGE (mV)

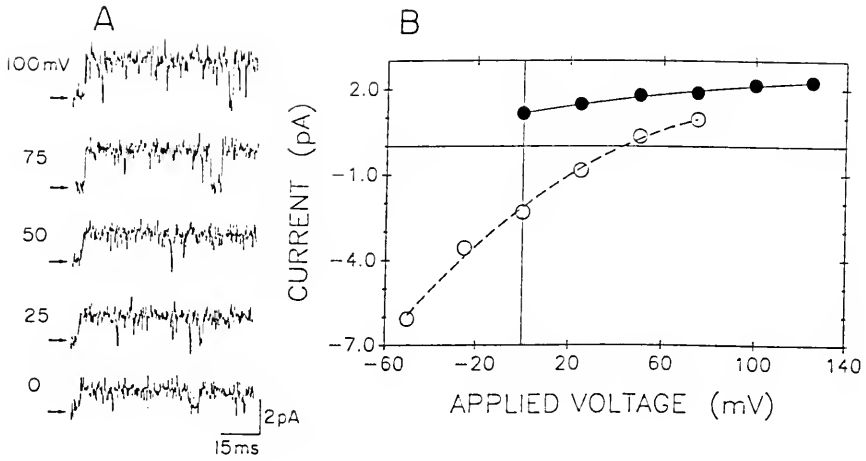
Fig. 3-10. Representative records showing the Ca^{++} dependence of the activation of a 215 pS K^{+} channel in an inside-out patch at -10 mV. Openings became longer and more frequent as the Ca^{++} concentration was increased from 10^{-7} M (top trace) to 10^{-6} M (bottom trace). Na^{+} patch solution was in the pipette and KCl saline was the intracellular solution. Arrows indicate the current level of the closed state.


4pA
5ms

A voltage-dependent K^+ channel

Depolarizing voltage steps applied to cell-attached patches often revealed a channel carrying outward current. Hyperpolarizing prepulses of -30 mV or -50 mV increased the frequency of channel openings but were not a necessary condition for activation of the channel. With Na^+ salines in the patch pipette, the slope conductance for outward currents was 9.7 ± 2.6 pS (N = 3). The slope conductance of the inward current through the cell-attached channel was 49.4 ± 14.8 pS with KCl saline in the patch pipette (N = 5). The inward rectification predicted by these slope conductances was small but apparent in the I-V relationships (Fig. 3-11A, B). Reversal potentials (in mV from the resting membrane potential) extrapolated from the most linear portions of I-V relationships were 45 ± 14 mV (N = 4) with 480 mM KCl applied extracellularly and -105 ± 14 mV (N = 3) with 480 mM NaCl applied extracellularly. These values suggest a selectivity for K^+ over Na^+. The activation of this channel by depolarizing voltage pulses was delayed. The first latencies of one channel activated by depolarizing pulses preceded by hyperpolarizing prepulses was 259 ± 147 ms at -25 mV from rest, 55 ± 89 ms at 0 mV from rest, and 27 ± 15 ms at 25 mV from rest. Channel activity sometimes occurred throughout the duration of depolarizations lasting as long as 5 sec. However, steady-state activity was never observed, leading to the conclusion that strong inactivation occurred, but with a slow onset. In inside-out patches held at -70 mV, channel activity was observed

Fig. 3-11. A voltage dependent, K^+ selective channel with properties of a delayed rectifier. A. Representative recordings of a channel in a cell-attached patch during activation by 5 s voltage steps from a holding potential of -30 mV from rest. The pipette contained Na^+ patch solution. Arrows indicate the current levels of the closed state. f_c = 1.5 kHz. B. The I-V relationships of two other channels in cell-attached patches with 480 mM KCl (open circles) and 480 mM NaCl (closed circles) in the pipette. Note the inward rectification displayed by this channel. The curves were fit to these data by nonlinear regression.


A
100mV
75
50
25
0
2pA
15ms
B
2.0
-1.0
-4.0
-7.0
CURRENT (pA)
-60
-20
20
60
100
140
APPLIED VOLTAGE (mV)

during voltage steps to -60 mV or above. Rare openings at -70 mV did occur at the offset of depolarizing voltage steps.

A steady state Cl^- channel

Also observed in cell-attached patches was a 35 ± 13 pS channel whose reversal potential was near the resting membrane potential (-8.2 mV ± 9.5 mV from rest, N = 9). This channel was relatively common, being found in 26 of 102 patches, and was selective for Cl^- over cations. The current-voltage relationships (Fig. 3-12A, B) of inside-out patches with 480 mM KCl on the extracellular face of the patch and 480 mM NaCl on the intracellular face, gave a mean reversal potential of -2.0 ± 1.8 mV (N = 5). In these inside-out patches the slope conductance was 70 ± 4 pS. In three channels in inside-out patches, intracellular perfusion with the Cl^- channel blocker potassium isothiocyanate (20 mM) reduced the slope conductance of the chloride channel by 45% and 57%, while the third channel was unaffected.

The Cl^- channel was active at all holding potentials tested and its P_o was relatively independent of the holding potential (Fig. 3-13). The distributions of the open dwell times were usually best fit by single exponentials, giving mean open times ranging from 1.1 to 6.9 ms. The distributions of the closed dwell times were better fit by two exponentials, with mean closed times ranging from 1.6 to 3.9 ms. Neither the mean open or closed times showed any consistent dependence on membrane potential.

Fig. 3-12. A steady state, Cl^- selective channel. A. Representative recordings of a Cl^- channel in an inside-out patch with KCl saline in the patch pipette and an intracellular solution of NaCl saline. Arrows indicate the current level of the closed state. f_c = 1.5 kHz. B. The I-V relationship of a another Cl^- channel in an inside-out patch under the same conditions. The slope of the linear regression fit to these data was 71 pS.

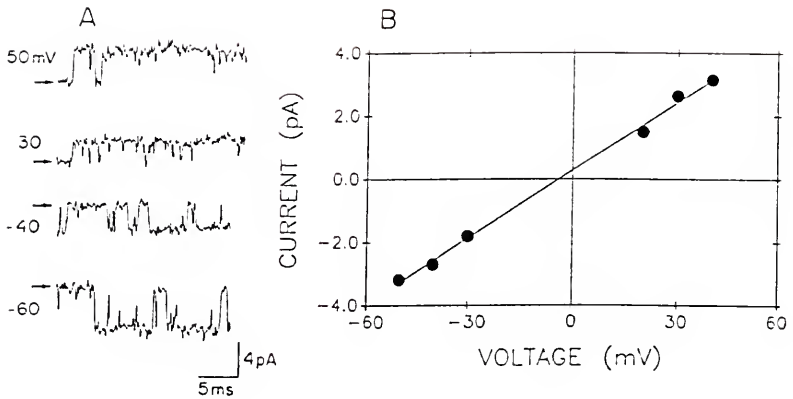
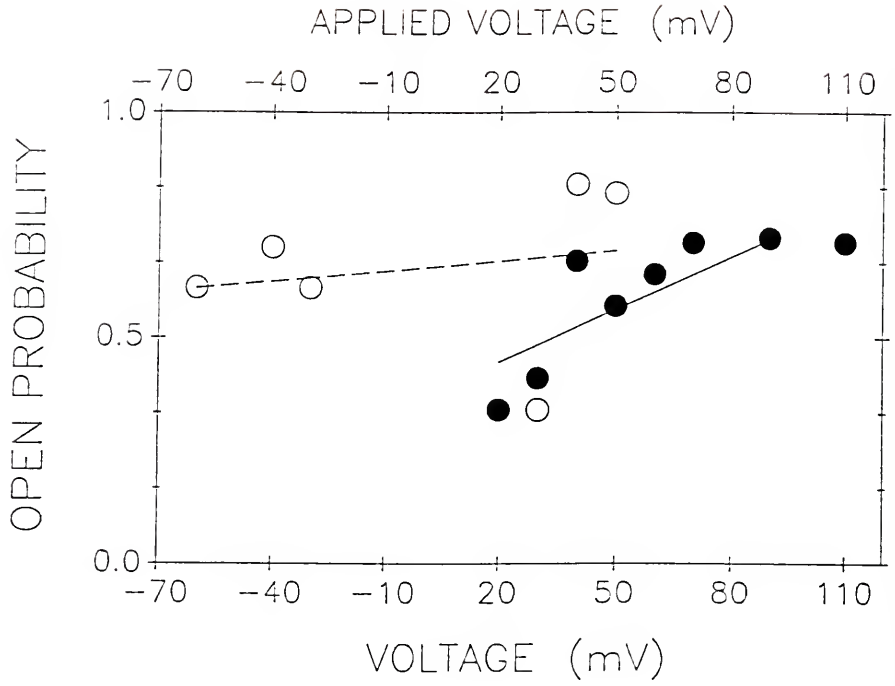

A
50mV
30
-40
-60
4pA
5ms
B
4.0
2.0
0.0
-2.0
-4.0
CURRENT (pA)
-60
-30
0
30
60
VOLTAGE (mV)

Fig. 3-13. Plot of the P_o of a Cl^- channel versus voltage. The P_o of this channel displayed little dependence upon voltage in either the cell-attached (closed circles) or the inside-out (open circles) configuration. These data were obtained from the same patch before and after excision. Lines were fit to the data by linear regression.


APPLIED VOLTAGE (mV)
−70
−40
−10
20
50
80
110
1.0
0.5
0.0
OPEN PROBABILITY
VOLTAGE (mV)

Discussion

The nonselective cation channel

The nonselective cation channel shares several properties with a channel found in Aplysia neurons (Chesnoy-Marchais, 1985): 1) it is resolved only in inside-out patches; 2) high internal concentrations of Na^+, but not Cs^+ or K^+, appear to prevent inactivation; 3) its kinetics are sensitive to the type of divalent cation present; 4) it is weakly voltage dependent; 5) it appears to have a multi-ion pore, and if it is permeable to divalent cations as my data suggest; 6) Ba^{++} gives a higher conductance than Ca^{++}; and 7) its current-voltage relationship shows inward rectification in high external concentrations of divalent cations, especially at potentials above E_{Na}.

Mg^{++} block offers a partial glimpse of the structure of the cation channel. The channel appears to have at least two binding sites for cations in its pore, and another divalent cation binding site outside the electric field of the membrane capable of altering the gating properties of the channel. This latter site may be very near the permeation pathway, however, since both internal and external permeable divalent cations decreased the P_o. Alternatively, a second, external binding site could exist. In addition to agreeing with the Woodhull model of channel block, the interpretation that fast block was due to Mg^{++} entering the pore is consistent with evidence that Na^+ and Mg^{++} compete for binding sites, namely that the dissociation constant for Mg^{++} depended on the direction of flow of Na^+ through the pore. When the holding

potential was above E_{Na} at 0 mV, where Na^+ should force internal Mg^{++} into the channel, the apparent dissociation constant was in the millimolar range. When the holding potential was below E_{Na}, where Na^+ should oppose the entry of internal Mg^{++} into the pore, the apparent dissociation constant was decreased 100-fold. This type of interaction between permeant ions is consistent with a multi-ion pore, but to prove this conclusion, future studies would need to show that Na^+ causes an increase in the dissociation rate constant of Mg^{++} (Moczydlowski, 1986). Further evidence for a multi-ion pore could also be obtained by investigating the mole fraction dependence characteristic of multi-ion pores (Hille and Schwarz, 1978). My data would predict a conductance minimum at about 10 mM divalent cation and 480 mM Na^+, a ratio of about 0.2.

If the channels permeable to divalent cations in inside-out patches were, in fact, nonselective cation channels, then the divalent cations replaced Na^+ as the major permeant ion. Ca^{++} or Ba^{++} can supersede monovalent cations as major permeant ions in channels when they bind more strongly to sites within the channel pore, effectively excluding the monovalent cation (Dani and Eisenman, 1987). A necessary corollary of this assertion is that Ca^{++} and Ba^{++} should have a lower conductances than Na^+. While my data do not directly address this point, Ba^{++}, which appeared to bind less strongly within the pore than Ca^{++} by virtue of its weaker blocking ability, gave a higher conductance than Ca^{++} under identical conditions. Furthermore, the properties of the fast block

by Mg^{++} strongly suggest that divalent cations bound more strongly to sites within the pore than monovalent cations.

Internal divalent cations caused reductions in the P_o by interacting with other sites apparently outside the electric field of the membrane. The effect appeared to be mediated by a site (or sites) on the channel protein and not by charge screening of the diffuse negative charges on the membrane (which alters the surface potential and effectively changes the profile of potential across the membrane). Charge screening by internal cations should cause a negative shift in the activation curve of voltage dependent channels (McLaughlin et al., 1971), whereas I observed a positive shift.

K^+ channels

The large conductance and Ca^{++} dependency of the Ca^{++}-activated K^+ channel is similar to channels from several types of vertebrate cells (Wong and Adler, 1986; Yellen, 1984; Pallotta et al., 1981). A similar 130 pS Ca^{++}-activated potassium channel has been reported in mouse olfactory receptor neurons (Maue and Dionne, 1987). The mouse and lobster channels are similar in their Ca^{++} concentration dependency, resistance to TEA, and mean open durations, but differ in their sensitivity to Cs^+ block.

The voltage-activated K^+ channel in lobster olfactory receptor cells is most similar to the delayed rectifier class of K^+ channels because of its delayed activation, slow inactivation and small unitary conductance. Although its increased activation following prehyperpolarization and its inward rectification are not properties of classical delayed rectifier currents, they are not inconsistent

with the heterogeneity among channels now classified as delayed rectifier channels (Rudy, 1988). A similar channel, but with a much larger slope conductance of 40 pS, was found in mouse olfactory receptor cells (Maue and Dionne, 1987). The channels from mouse and lobster olfactory receptor cells both show slow inactivation and similar first latencies.

Chloride channel

The steady state Cl^- channel appears to provide the receptor cell with a constant leak pathway for Cl^-, suggesting that the lobster olfactory receptor cell has a passively distributed concentration gradient of Cl^-. That the channel's reversal potential in cell-attached patches was close to the resting membrane potential agrees with this conclusion. Block of the channel by isothiocyanate, which is a permeant anion in some Cl^- channels (Franciolini and Nonner, 1987), agrees with the isothiocyanate block of the voltage-gated Cl^- channel from Torpedo electric organs (Tank et al., 1982). A voltage-dependent Cl^- channel of larger conductance has been described in mouse olfactory receptor neurons (Maue and Dionne, 1987).

Significance for olfaction

The two K^+ channels in lobster olfactory receptor neurons appear to have counterparts in vertebrate olfactory receptor cells (Maue and Dionne, 1987; Firestein and Werblin, 1987; Trotier, 1986); all probably function in membrane repolarization. They probably also underlie the outward rectification observed in lobster olfactory receptor cells (Schmiedel-Jakob et al., 1989), which may

help to limit the magnitude of odor-evoked depolarizations to the voltage range over which the cell is capable of producing spikes. Unlike mouse and salamander olfactory receptor cells, however, lobster receptor cells do not appear to have inward rectifier K^+ channels, believed to contribute to the stability of the membrane potential (Maue and Dionne, 1987; Trotier, 1986). This function could be subserved in lobster olfactory receptor cells by the steady state Cl^- channel. This Cl^- channel may also be partly responsible for the lower input resistance of lobster olfactory receptor cells (Schmiedel-Jakob, 1989) compared to that of salamanders (Firestein and Werblin, 1987; Trotier, 1986).

The function of the cation channel, however, remains a mystery. Conceivably, it could enhance the spread of odor-evoked depolarizations by summing its inward current with the odor-evoked inward currents, effectively increasing the space constant of the cell (Yoshii et al., 1988). Positive feedback would be avoided if the nonselective cation current was less than the outward leak current. The lack of any convincing evidence that it is active in intact cells and its suppression by Mg^{++}, however, suggest that the channel is unlikely to be active under normal physiological conditions. Similar channels in molluscs appear to be activated only by physiologically stressful conditions. Strong et al. (1987) observed this type of channel in cell-attached patches from Aplysia neurons, but only following injury to the processes of these cells. Yazejian and Byerly (1989) recently recorded a similar channel in Helix neurons and found that it could be activated by sustained

intracellular perfusion with high Ca^{++} solutions or low Ca^{++} solutions lacking ATP, or by a low Ca^{++}, high K^{+} bath solution. Since my data come from cells bathed in low divalent saline, which is probably stressful to the cells, it is possible that this species of cation channel is also involved in responses to trauma in the lobster. Trauma-induced resorption of processes might occur regularly in neurons whose dendrites are exposed to damage as are those of olfactory receptor cells.

CHAPTER 4
DOES cAMP MEDIATE OLFACTORY TRANSDUCTION?

Introduction

The binding of odorants to receptors in the exposed membranes of olfactory receptor cells initiates the conversion of chemical information into electrical signals, a process called olfactory transduction. The study of olfactory transduction has advanced rapidly by investigating mechanisms of transduction previously discovered in postsynaptic and photoreceptor cells. Earlier evidence that volatile odorants might stimulate a cyclic nucleotide second messenger system in vertebrate olfactory receptor cells (Kurihara and Koyama, 1972; Dodd and Persaud, 1981) has been substantiated by measurements of adenylate cyclase activity in olfactory cilia from frogs and rats (Pace et al., 1985; Sklar et al., 1986; Shirley et al., 1986). Physiological evidence provides further support for this mechanism. The perfusion of cAMP into salamander olfactory receptor cells evokes an inward current (Trotier and MacLeod, 1987), and both cAMP and cGMP activate a nonselective cation channel in excised patches from frog olfactory cilia (Nakamura and Gold, 1987). Extracellular superfusion with 8-bromo-cAMP mimics odorant-evoked currents in voltage-clamped olfactory mucosa from bullfrogs (DeSimone et al., 1988). A cAMP-dependent conductance from homogenates of rat olfactory epithelium

has also been successfully incorporated into planar lipid bilayers (Vodyanoy and Vodyanoy, 1987). Although the evidence for cyclic nucleotides as a transduction mechanism in olfaction is impressive, proof that changes in cyclic nucleotide concentration are necessary for odor responses is still lacking. Evidence, however, has also been obtained for other mechanisms of transduction in vertebrate olfactory receptor cells. Amino acids stimulate phosphoinositide turnover in catfish olfactory cilia (Huque and Bruch, 1986), presumably via a GTP-binding protein and phospholipase C characterized from this tissue (Bruch and Kalinowski, 1987; Boyle et al., 1987). A novel odorant-gated channel has also been reported in recordings of ion channels from frog olfactory cilia reconstituted in planar bilayers (Labarca et al., 1988). Furthermore, comparative aspects of olfactory transduction, including possible similarities across taxonomic boundaries and the possible specificity of transduction mechanisms for types of odorants, have yet to be investigated.

In contrast to vertebrates, evidence for transduction mechanisms in invertebrate olfactory receptor cells is virtually absent. Early evidence implicating cyclic nucleotides in transduction in insect pheromone receptor cells (Villet, 1978) has not been substantiated (Ziegelberger, 1988). Given the wealth of evidence for the production of cAMP as a mechanism of olfactory transduction in vertebrates, I chose to investigate the hypothesis that cAMP mediates olfactory transduction in olfactory receptor cells of the spiny lobster. Both biochemical and physiological

experiments failed to support the hypotheses that odorants stimulate production of cAMP, or that cAMP is involved in generating receptor potentials. In a few instances, a phosphodiesterase inhibitor, alone and in combination with other drugs, enhanced the amplitude of the receptor potential, but in a manner more consistent with changes in modulation or adaptation of odor sensitivity than with transduction.

Methods

Preparation

Specimens of the spiny lobster (Panulirus argus) were collected in the Florida Keys and maintained in flowing seawater at 17 to 21°C. The paired olfactory organ of lobsters consists of a tuft of hair-like sensilla (aesthetascs) located on the lateral filament of the antennule (see Fig. 1-1). Ultrastructural evidence indicates that the hairs contain only the branched outer dendritic segments of the olfactory receptor cells, which extend to nearly the tip of the hair, and inner dendritic segments and auxiliary cell processes, which end in the base of the hair (see Fig. 1-2). The somata of the olfactory receptor cells are clustered in the lumen of the antennule. Hydrophilic compounds applied to hairs probably have access only to the outer dendritic segments of the olfactory receptor neurons.

Radioimmunoassay

The cAMP content of the outer dendritic segments was measured by radioimmunoassay (RIA) in an experiment where the cAMP content of the stimulated hairs from one antennule of a lobster was compared to

the unstimulated hairs on the other antennule. A live lobster was restrained and one of the antennules was suspended in a stirring saline solution (Trapido-Rosenthal et al., 1989) containing drugs and/or odorants. The other antennule was similarly exposed to a control solution. I compared the effect of 50 μM forskolin or one of three odorants, in the presence of a phosphodiesterase inhibitor, to the effect of the phosphodiesterase inhibitor alone on the cAMP content of the hairs. For experiments using adenosine 5'-monophosphate as the odorant, papaverine hydrochloride was the phosphodiesterase inhibitor (Clark et al., 1974; Londos et al., 1981). For the other odorants, TetraMarin extract and a mixture of identified stimulatory molecules, IBMX was the phosphodiesterase inhibitor. The effect of 1 mM IBMX was also compared to saline. After a 1 min (or as noted) exposure, each of the antennules was wiped dry and the hairs were rapidly scraped off and placed into 220 μl of 0.1 N HCl. In order to mimic the lobster's normal pattern of periodically sampling the odorant environment (Schmitt and Ache, 1979), the antennules of some restrained lobsters were stimulated by an alternative method. A pneumatically driven valve was used to inject 20 μl of saline containing odorants and/or drugs every 5 s for 2 min into a stream of control solution that continuously flowed over one of the antennules at 4 ml/min. In these trials, TetraMarin extract was used as the odorant. Again, the lobster's other antennule served as a control. Tissue was processed for RIA of cAMP according to Kaupp et al. (1982). The amounts of cAMP in samples from both experiments were determined using a commercial RIA kit

with acetylation (Biomedical Technologies Inc., Cambridge, MA). Protein was determined by a modified Lowry method (Petersen, 1977). Values are reported as means ± 1 standard deviation. Student's paired t-tests were performed according to Steel and Torrie (1980).

Intracellular recording

Hemisections containing the olfactory receptor cells were cut from an antennule and prepared for intracellular recording using the whole-cell patch technique as described previously (Chapter 1). Single hemisections were wedged tightly into a slit separating two compartments of a silicon elastomer (Sylgard, Dow Corning, Midland, MI) chamber. The hairs projected downward into a tubular compartment, allowing the hairs to be superfused continuously with saline at 3.0 to 3.5 ml/min. Saline containing drugs or odorants was introduced into the flow by two rotary injection valves (Rheodyne 5020). The first valve contained a 1 ml loop which was used to introduce membrane-permeable drugs. Dye injections showed that the solution in the 1 ml loop required about 5 s to reach the hairs and about 70 s to clear. The second valve contained a 20 µl loop which was used to introduce odorants. Dye injections showed that the odorant solution required 2 s to reach the hairs and about 5 - 6 s to clear. The lumen of the hemicylinder, which is literally filled with the somata of the olfactory receptor cells, faced upward into the main compartment of the chamber, which formed a static saline bath and allowed direct access to the somata for recording.

Whole-cell recording using patch-clamp techniques allowed simultaneous current injection and voltage recording. Gigaohm seals

were formed between the somatic membrane of olfactory receptor cells and borosilicate glass pipettes (Jencons Scientific, Leighton Buzzard, England) pulled to a tip diameter of about 1 μm (bubble numbers of 4.6 to 5.4, Mittman et al., 1987). After compensating the capacitance of the electrode, the whole-cell recording configuration was achieved using suction to break through the cell membrane, and the current clamp mode was selected. A commercial amplifier with a 1 gigaohm head stage (Dagan Instrument Co., Minneapolis, MN) was used and recordings of membrane voltage were stored on analog video tape after being low-pass filtered at 10 kHz (Bezanilla, 1985). The resting membrane potential at the recording site in the soma was maintained at -65 to -75 mV by current injection using a balanced Wheatstone bridge circuit. Recordings were made at room temperature, which varied from 22 to 24°C.

Solutions and chemicals

The composition of saline was (mM) 460 NaCl, 13 KCl, 13 $CaCl_2$, 10 $MgCl_2$, 1.7 glucose, 3 HEPES, pH 7.4. The composition of the patch solution was (mM) 200 KCl, 2 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 570 glucose, 10 HEPES, 34 NaOH, pH 7.4. Stock solutions of odorants consisted of (1) AMP, (2) MIX - equimolar mixture of 9 identified stimulatory molecules (Derby and Ache, 1984a) that included L-glutamate, taurine, betaine, trimethylamine oxide, glycine, L-glutamine, L-phenylalanine, L-alanine, DL-lactate, and (3) TET - an extract of TetraMarin, a commercial fish food (TetraWerke, Melle, FRG). TET was prepared by dissolving 2 mg of TetraMarin in 60 ml saline, centrifuging at 1400 x g and filtering the supernatant

through Whatman #3 filter paper. A 50 mM stock solution of forskolin (Calbiochem, La Jolla, CA) was prepared in ethanol and stored at 0°C. Inorganic salts were purchased from Fisher Scientific (Fair Lawn, NJ) and HEPES and EGTA were purchased from Research Organics (Cleveland, OH). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Radioimmunoassay

The amount of cAMP measured in samples from olfactory organs stimulated by the continuous and periodic methods of application fell within the same range and were pooled for analysis. 50 μM forskolin plus 1 mM IBMX (Fig. 4-1, FOR) increased the level of cAMP to 328 ± 126 % (N = 5) of the IBMX control, a statistically significant difference ($p < 0.01$, 1-tailed, 4 df). 1 mM IBMX alone (Fig. 4-1, IBMX) increased the level of cAMP to 170 ± 56 % (N = 6) of the saline control, also a significant difference ($p < 0.025$, 1-tailed, 5 df).

All three odorants, however, failed to stimulate the production of cAMP above their phosphodiesterase inhibitor controls. A 1:100 dilution of TET plus 1 mM IBMX (Fig. 4-1, TET1) yielded 91 ± 41 % (N = 12) of the level of cAMP in the IBMX controls, not a significant difference ($p > 0.5$, 2-tailed, 11 df). Preincubation in 1 mM IBMX for 3 min prior to exposure to TET plus IBMX for 1 min (Fig. 4-1, TET-P) also resulted in no change, yielding 90 ± 28 % (N = 3) of the IBMX control level of cAMP ($p > 0.5$, 2-tailed, 2 df). Similarly, increasing the duration of

incubation had no effect on the level of cAMP. Incubations with TET plus IBMX for 10 and 20 min (N = 2 per time point) resulted in cAMP levels that were 99 ± 25 % (Fig. 4-1, TET10) and 78 ± 55 % (Fig. 4-1, TET20), respectively, of the IBMX control. The cAMP level in hairs incubated for 5 min in 0.1 mM MIX plus 1 mM IBMX were 94 ± 30 % (N = 5) of the level of cAMP of the IBMX control (Fig. 4-1, MIX), not a significant difference ($p > 0.5$, 2-tailed, 4 df). The level of cAMP in response to 5 min applications of 0.5 mM AMP plus 0.5 mM papaverine (Fig. 4-1, AMP) was 108 ± 25 % (N = 5) of the papaverine control, also not a significant difference ($p > 0.5$, 2-tailed, 4 df).

The amount of cAMP in tissue exposed to saline only was 9.1 ± 9.2 fmoles/μg protein. The increase in cAMP evoked by IBMX suggests that the mean basal activity of adenylate cyclase in the tissue was 3.9 ± 2.3 pmoles/mg protein/min (N = 5). While both adenylate cyclase and phosphodiesterase activity is present in the hairs, the biochemical data provide no evidence to support the assertion that adenylate cyclase was stimulated by the odorants used.

Intracellular recording

The membrane potentials of all 42 olfactory receptor cells tested were unaffected by superfusion of the outer dendritic segments with one or more of the following: 50 μM forskolin plus 1 mM IBMX (N = 27; Fig. 4-2), 1 mM IBMX (N = 19), 50 μM forskolin (N = 6), 1 mM 8-bromo-cyclic guanosine 3'-5'monophosphate (8-bromo-cGMP) plus 1mM IBMX (N = 4), and 1 mM 8-bromo-cAMP plus 1 mM IBMX (N = 4).

Fig. 4-1. Plot of the percent change in the amount of cAMP found in lobster olfactory hairs following exposure to odorants and/or pharmacological agents, compared to the amounts of cAMP generated by their respective control solutions (100 %). FOR = forskolin; IBMX; TET1, TET10, TET20 = TetraMarin extract plus IBMX for 1, 10, and 20 min, respectively, without IBMX preincubation; TET-P = TET plus IBMX for 1 min with 3 min preincubation in 1 mM IBMX; AMP; MIX = mixture of stimulatory odorants as described in Methods. FOR, TET1, TET10, TET20, TET-P, and MIX are compared to IBMX in saline. AMP is compared to papaverine hydrochloride in saline. IBMX is compared to saline. * = statistically significant (see Text).

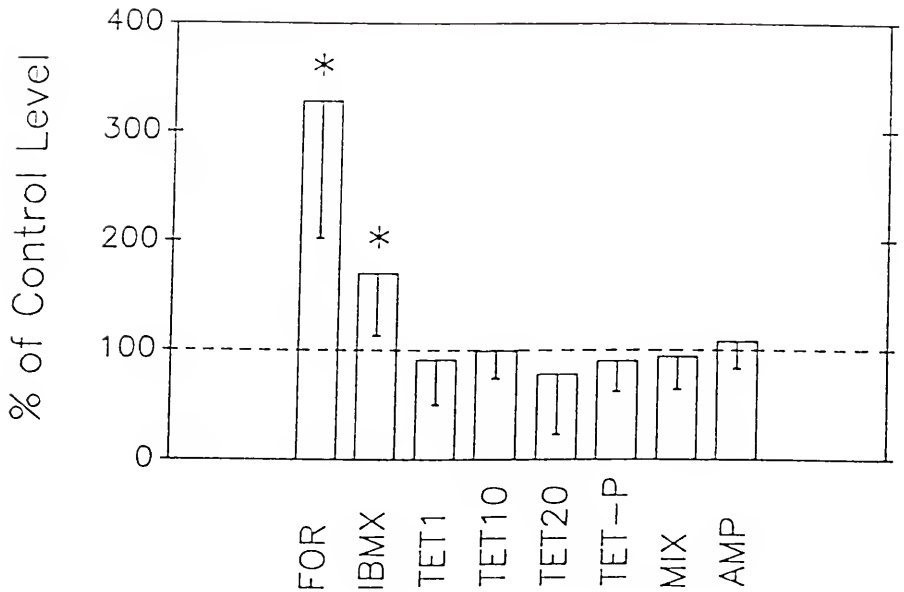
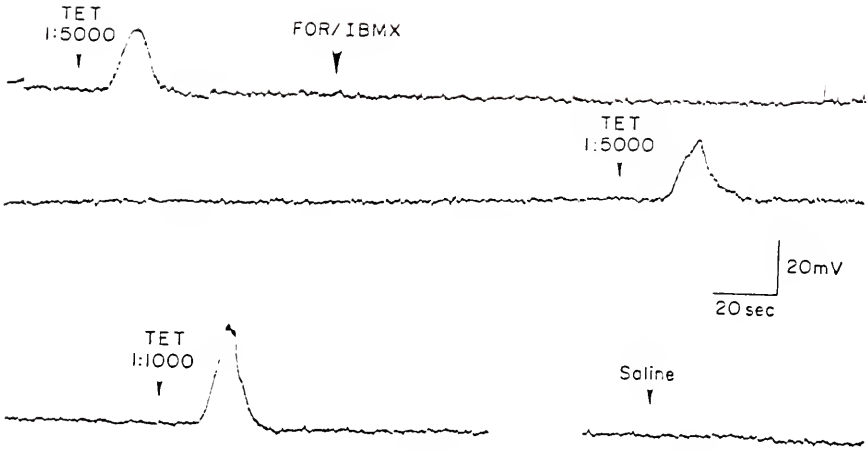

% of Control Level
400
300
200
100
0
*
*
FOR
IBMX
TET1
TET10
TET20
TET-P
MIX
AMP

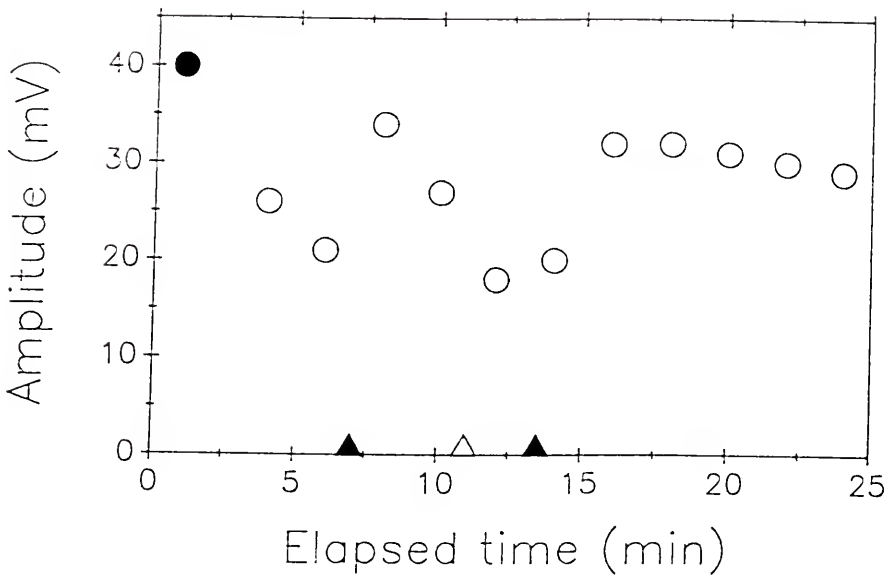
Fig. 4-2. Intracellular records from a lobster olfactory receptor cell showing (upper two traces) the absence of a response to 50 μM forskolin plus 1 mM IBMX in saline (FOR/IBMX) superfused over the outer dendritic segments. The response to a 1:5000 dilution of TET evoked before superfusion of forskolin plus IBMX (top trace) was unchanged (middle trace) by exposure to these drugs. Superfusion of a 1:1000 dilution of TET (lower trace) indicated that the cell was capable of generating larger depolarizing receptor potentials. Saline evoked no response (lower trace). Holding potential = -68 mV. The upper two traces are continuous.


TET
1:5000
FOR/IBMX
TET
1:5000
20mV
20sec
TET
1:1000
Saline

Similarly, the amplitude of the receptor potential evoked by TET following superfusion of a drug was unchanged relative to the amplitude before exposure to the drug in 36 of the 42 cells. In all 36 cells, the amplitude of receptor potential remained within a few millivolts of the initial value for 15 - 25 min post exposure, the duration of a typical recording. In each instance, the dilution of TET applied was adjusted to evoke a receptor potential of 15 - 25 mV, which is approximately half the maximum amplitude of receptor potentials (40 - 50 mV) observed in the soma of these cells (Schmiedel-Jakob et al., 1989). This dilution was then applied at 1 or 2 min intervals for the duration of the recording.

In 6 of the cells, some of which received multiple treatments, however, the amplitude of the receptor potential increased within 1 - 3 min following superfusion with forskolin plus IBMX (N = 4), IBMX (N = 2), and 8-bromo-cGMP plus IBMX (N = 2) and recovered within several minutes thereafter. One of these cells is depicted in Fig. 4-3. The average increase in amplitude in response to the first drug application for all treatments was 61 $\pm$ 45 % (N = 6) of the mean response prior to drug application, a significant difference ($p < 0.05$, 1-tailed, 5 df). The effect was repeatable in the three cells in which it was possible to test the drugs at least twice, although recovery from the second drug application was prolonged relative to the first. The lack of any direct effect of the forskolin-IBMX mixture, whose ability to enter the tissue in the hairs and increase the level of cAMP was demonstrated in the biochemical experiments, however, argues against the possibility that transduction was mediated by cAMP in these six cells.

Fig. 4-3. Plot of the peak amplitude of receptor potentials from a lobster olfactory receptor cell vs. elapsed time. The amplitude of a receptor potential evoked by superfusing the outer dendritic segments with a 1:500 dilution of TET (filled circle) demonstrates a nearly maximal response. The amplitude of receptor potentials evoked by a 1:10000 dilution of TET (open circles) applied at 2 min intervals increased following superfusion with 50 μM forskolin plus 1 mM IBMX (closed triangles) but not by superfusion with a saline blank (open triangle).


Amplitude (mV)
40
30
20
10
0
0
5
10
15
20
25
Elapsed time (min)

Discussion

In contrast to the affects of odorants on vertebrate olfactory cilia, these results provide no evidence that the odorant, TET, stimulated the production of cAMP in the outer dendritic segments of lobster olfactory receptor cells. The results with TET are consistent with the biochemical experiments using the other two odorants, MIX and AMP. The relatively low adenylate cyclase activity measured in lobster hairs, compared to vertebrate olfactory cilia, is perhaps consistent with the lack of effect of odorants. In vertebrate olfactory cilia where odorants do stimulate the production of cAMP, the basal adenylate cyclase activity was 100 to 1000 times higher than what I measured in the lobster (Sklar et al., 1986; Shirley et al., 1986).

The odorants used have been shown to be adequate stimuli for lobster olfactory receptor cells (Derby and Ache, 1984a,b; Derby et al., 1984; Schmiedel-Jakob et al., 1989). The use of a broad-spectrum, complex odorant (TET) allowed me to test many individual compounds simultaneously. Obtaining similar results with AMP and the mixture of nine identified odor compounds (MIX) reduced the unlikely possibility that the complex mixture obscured positive responses from a few component compounds in the biochemical assays.

My biochemical results are consistent with the lack of any physiological effect of pharmacological agents known to mimic cAMP or to enhance the accumulation of cAMP. If stimulation of adenylate cyclase were a mechanism of olfactory transduction in the cells from which I recorded, then application of forskolin and

IBMX, or of 8-bromo-cAMP should have depolarized the cells directly. The ability of these compounds to evoke physiological responses directly has been demonstrated in other systems (e.g. DeSimone et al., 1988; Pollock et al., 1985). Furthermore, the production of cAMP is stimulated better by forskolin than by odorants even in vertebrate olfactory tissue (Sklar et al., 1986). The ability of forskolin and IBMX to stimulate the accumulation of cGMP in lobster nerve-muscle preparations (Goy et al., 1984), suggests that many lobster olfactory receptor cells lack any mechanism for cGMP, as well as for cAMP, to produce electrical responses. The latter conclusion is further supported by the lack of any direct effect of 8-bromo-cGMP in my recordings.

That membrane-permeable agents repeatably enhanced receptor potential amplitude in 6 of 42 cells, by itself, could be interpreted as supporting the hypothesis that cAMP mediates transduction in lobster olfactory receptor cells. Three reasons counter this interpretation, however. First, membrane-permeable agents failed to cause direct responses or adaptation of the odor response as would be expected if cAMP mediated transduction. Second, only the amplitude, not the time course of the receptor potential was affected. The presence of a phosphodiesterase inhibitor would block the recovery of odor responses if recovery depended on the removal of the cyclic nucleotide. Conversely, if recovery was due to adaptation triggered directly by cAMP, then the response should have recovered more quickly than normal. Third, the biochemical experiments failed to demonstrate an odor-evoked

increase in cAMP. I conclude, therefore, that the enhanced responses in these six cells did not reflect the drugs mimicking a transduction mechanism. Other studies support this conclusion. The conclusion that enhanced responses to pheromones observed after perfusing moth antennae with phosphodiesterase inhibitors and membrane-permeable analogs of cyclic nucleotides is evidence for transduction by cyclic nucleotides (Villet, 1978), conflicts with recent biochemical evidence that cyclic nucleotides might be involved in adaptation processes or other functions in moth antennae (Ziegelberger, 1988). Phosphodiesterase inhibitors, forskolin and cAMP analogs sometimes increased odor-evoked responses in frog olfactory mucosa (DeSimone et al., 1988), where biochemical studies conclusively demonstrate that odorants activate adenylate cyclase (Pace et al., 1986; Sklar et al., 1986; Shirley et al., 1986), but these compounds also mimicked odor responses directly, evoked a standing current, and caused reduction of the odor response, as would be expected if cyclic nucleotides mediated transduction. The similarity of my results to those of the moth raises the intriguing possibility that differences in mechanisms of olfactory transduction may follow taxonomic boundaries.

Physiological evidence could also be obtained by intracellular perfusion of the cells with substances which mimic, stimulate or inhibit second messenger systems (e.g. Trotier and MacLeod, 1987). While these experiments have been attempted with consistently negative results (McClintock and Ache, unpublished data), one must allow that the substances failed to reach the outer dendritic

segments within the duration of the intracellular recordings. The lobster olfactory receptor cell has the longest and thinnest dendritic arbor yet reported for any olfactory receptor cell (Grünert and Ache, 1988), which must be a significant impediment to diffusion.

In summary, my results provide little evidence to suggest that cyclic nucleotides are involved in transduction of responses to a complex odorant in lobster olfactory receptor cells. Because failure to disprove the null hypothesis does not constitute positive proof, cyclic nucleotides must remain candidate transduction mechanisms, albeit less likely ones, in lobster olfactory receptor cells. It is conceivable that odorant molecules not contained in TET could prove to activate lobster olfactory receptor cells by mechanisms involving cyclic nucleotides. Future experiments investigating other potential mechanisms such as ligand-gated channels similar to those reported in taste cells (Hatt and Franke, 1987; Teeter and Brand, 1987) and odorant-stimulated phosphatidylinositol turnover as reported in catfish olfactory cilia (Huque and Bruch, 1986) may provide more positive results.

CHAPTER 5
HYPERPOLARIZING RECEPTOR POTENTIALS

Introduction

Inhibition of receptor cells by odorants is a poorly understood phenomenon. Its role in olfactory coding could be significant, yet we lack convincing evidence that odors inhibit olfactory receptor cells. Extracellular recording has long shown that some odors suppress the characteristically low level of spontaneous activity of vertebrate olfactory receptor cells (Sicard and Holley, 1984; Getchell and Shepherd, 1978a,b; Duchamp et al., 1974; Mathews, 1972; O'Connell and Mozell, 1969). Similar observations have been made for crustacean primary chemosensory neurons (Derby and Harpaz, 1988; Ache, unpublished observations). However, the conclusion that suppression of spiking observed in extracellular recordings reflects the activation of a specific inhibitory conductance is controversial (review: Getchell, 1986). For example, suppressed discharges in extracellular, multifiber records from amphibian olfactory receptor cells were recently proposed to have resulted from activity-induced changes in ion gradients among the tightly packed, unmyelinated afferent fibers (Gesteland and Adamek, 1987).

Intracellular records from amphibian (Getchell, 1977; Trotier and MacLeod, 1983; Trotier, 1986; Anderson and Hamilton, 1987) and crustacean (Anderson and Ache, 1985; Schmiedel-Jakob et al., 1989)

olfactory receptor cells, although still limited in number, reveal only depolarizations evoked by odors. Suzuki (1977; 1982) reported hyperpolarizations in lamprey olfactory receptor cells, but these were infrequent relative to depolarizations, they occurred in cells which were in a depolarized state, and were evoked only by high concentrations of odorant (10 mM). Maue and Dionne (1987) recorded odor-evoked outward currents in cell-attached patches on mouse olfactory receptor cells, but they caution that these currents could have been secondary responses to odor-activated inward currents.

This study found that graded, hyperpolarizing receptor potentials can be readily recorded in the somata of lobster olfactory receptor cells. Moreover, the same cells can also be depolarized by other odorants. The coexistence of excitatory and inhibitory responses to odors has important implications for understanding both olfactory transduction and peripheral mixture suppression.

Methods

Adult, intermolt specimens of P. argus were collected in the Florida Keys and maintained in flowing seawater at 18 to 23°C. Similar specimens of the American lobster, Homarus americanus, were purchased from commercial suppliers in Woods Hole, MA and maintained in flowing seawater at 13 to 17°C.

Olfactory receptor cells in antennular hemisections from P. argus were prepared for whole-cell recording according to Chapter 4, while those from H. americanus were prepared according to Anderson

and Ache (1985). Single hemisections were wedged tightly into a slit separating two compartments of a silicon elastomer (Sylgard from Dow Corning, Midland, MI) chamber. The hairs (aesthetascs) projected downward into a tubular compartment that was continuously superfused with saline at 3.5 to 4.0 ml/min. Odorants could be selectively applied to the hairs by injection of 20 µl volumes via a rotary injection valve (Rheodyne 5020) into the superfusing flow. Ultrastructural evidence indicates that the only neural elements in the hairs to which odorants have access are the outer dendritic segments of the olfactory receptor cells (Grünert and Ache, 1988). Odorants were applied at least 1 min apart to prevent adaptation, and were applied in order of increasing concentration when investigating dose-response relationships. The lumen of the hemicylinder, literally filled with the somata of the olfactory receptor cells, faced upward into the second compartment, a static saline bath that allowed direct access to the somata for recording. After successful recording sessions, both chambers were tested for leak by repeatedly injecting 1 M $CoCl_2$ into the superfusate and collecting the bath solution, and then by filling the bath with 1 M $CoCl_2$ and collecting the superfusate for 10 min. H_2S was bubbled through the collected fractions to produce insoluble, black CoS. This method could detect at least 0.1 mM $CoCl_2$.

Isolated somata were used in a few experiments for voltage-clamp recording. A single trituration step was used to remove somata from the enzyme-treated hemisections and to disperse the somata into a plastic culture dish. Odorants were pressure ejected

directly onto the isolated somata from glass pipettes with 1 μm tips.

Gigaohm seals were formed between the somatic membrane of the olfactory receptor cells and borosilicate glass pipettes (Boralex, Rochester Scientific, Rochester, NY or Jencons, Jencons Scientific, Leighton Buzzard, England) pulled to a tip diameter of about 1 μm (bubble numbers of 4.6 to 5.4, Mittman et al., 1987). Sylgard was applied to the neck of the pipette to reduce capacitance. Methods for current-clamp and voltage-clamp recording were described in Chapter 4 and Chapter 2, respectively.

The composition of lobster saline was (mM) 460 NaCl, 13 KCl, 13 $CaCl_2$, 10 $MgCl_2$, 1.7 glucose, 10 HEPES, pH 7.4. The composition of the patch solution was (mM) 26 KCl, 180 K acetate, 2 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 570 glucose, 10 HEPES, 34 NaOH, pH 7.0. Odorants consisted of (1) an extract of a commercial food for marine fish (TetraMarin from TetraWerke, Melle, FRG), 2 mg dissolved in 60 ml saline, centrifuged at 1400 x g and filtered through Whatman #3 filter paper, (2) a mixture of 1 mM L-proline, 1 mM L-arginine, and 1 mM L-cysteine, amino acids previously identified as suppressive components of a complex odorant for the spiny lobster (Derby and Ache, 1984a) and (3) 100 μM histamine. Odorants were prepared fresh daily or diluted from frozen stock solutions, adjusted to pH 7.4 and serially diluted with saline to the reported concentrations.

Results

All cells included in this report were depolarized by dendritic application (injection into the aesthetasc superfusion) of

TetraMarin extract (TET), which served as the search stimulus. None responded to dendritic application of saline blanks. Dendritic application of the three-component amino acid mixture (AAM), in contrast to TET, frequently evoked hyperpolarizing receptor potentials in cells from both species of lobsters. In H. americanus, 10 cells were hyperpolarized (Fig. 5-1) and six cells were unaffected by AAM. In P. argus, three cells were hyperpolarized (Fig. 5-2), three cells were depolarized and six cells were unaffected by AAM. The amplitudes of hyperpolarizing responses to AAM were dose-dependent for any one cell (Figs. 5-1 and 5-3), and varied among cells from -15 mV to -3 mV for 0.1 mM AAM. AAM and TET applied simultaneously to the dendrites (N = 2), virtually abolished the strong, depolarizing receptor potential otherwise evoked by TET (Fig. 5-4). Hyperpolarizing responses were not simply a general feature of amino acid stimulation because dendritic application of a mixture of six other amino acids (L-alanine, L-glutamate, L-glutamine, glycine, L-phenylalanine, and taurine), betaine, trimethylamine oxide, AMP, ADP, ATP either evoked no response (N = 5) or depolarized (N = 2) the cells.

In none of the preparations was there detectable leak between the two compartments of the recording chamber. The hyperpolarizations, therefore, were unlikely to result from activation of receptors on the soma or inner dendritic segment not associated with odorant reception. This possibility was further tested by pressure ejecting AAM directly onto isolated, voltage-clamped somata of H. americanus olfactory receptor cells. None of

Fig. 5-1. Current clamp record from a H. americanus olfactory receptor cell. Top trace: No response was evoked by injection of a saline blank. Second trace: Depolarization and spiking evoked by a 1:100 dilution of fish food extract (TET). Third trace: Hyperpolarization evoked by a 100 μM mixture of L-proline, L-arginine, and L-cysteine (AAM). Fourth trace: Small hyperpolarization evoked by 10 μM AAM. Bottom trace: Lack of response to 1 μM AAM. Odorants injected at arrow; the delay of approximately 2 s reflects the time required for the stimulus to reach the dendrites in this and all subsequent records. Membrane potential for all traces was -60 mV.

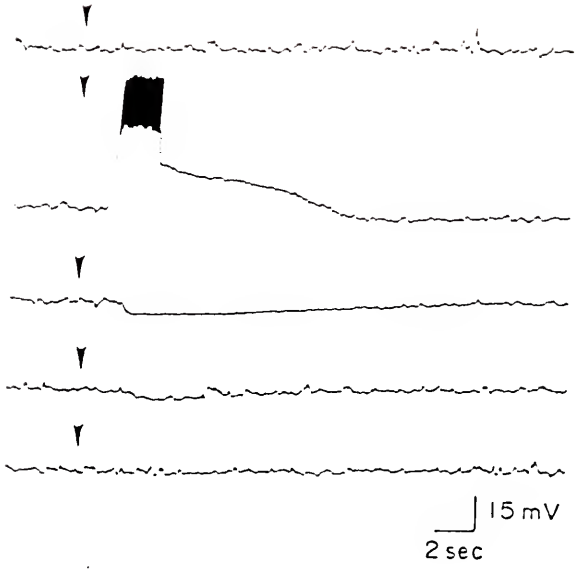

15 mV
2 sec

Fig. 5-2. Current clamp records from a P. argus olfactory receptor cell. Top trace: No response was evoked by injection of a saline blank. Membrane potential = -70 mV. Second trace: Depolarization evoked by a 1:100 dilution of TET. Membrane potential = -70 mV. Third trace: Hyperpolarization evoked by 100 μM AAM. Membrane potential = -47 mV. Bottom trace: Lack of response to 10 μM AAM. Membrane potential = -48 mV. Odorants injected at arrow.

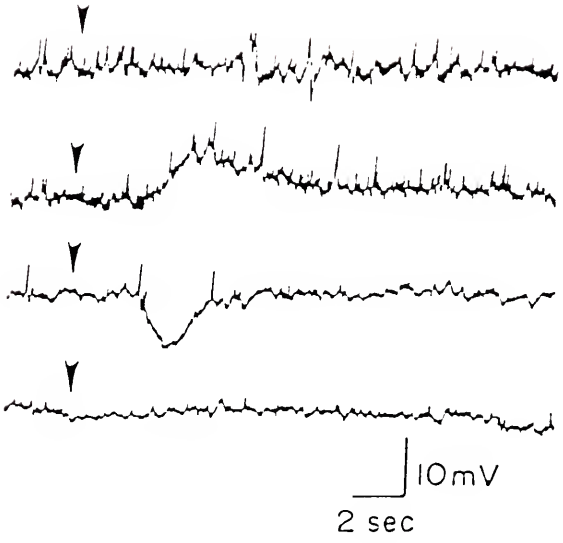

10mV
2 sec

Fig. 5-3. Current-clamp records from an American lobster receptor cell. Top trace: No response was evoked by injection of a saline blank. Second trace: A 15 mV hyperpolarization evoked by 100 μM AAM Third trace: A 13 mV hyperpolarization evoked by 10 μM AAM. Bottom trace: A 4 mV hyperpolarization evoked by 1 μM AAM. Odorants injected at arrow. Membrane potential for all traces was -68 to -70 mV.

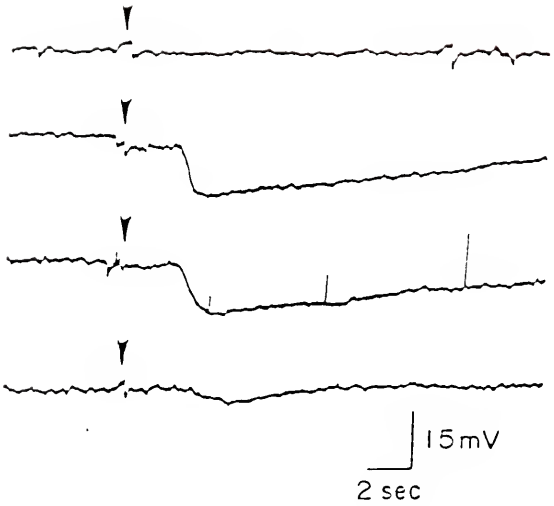
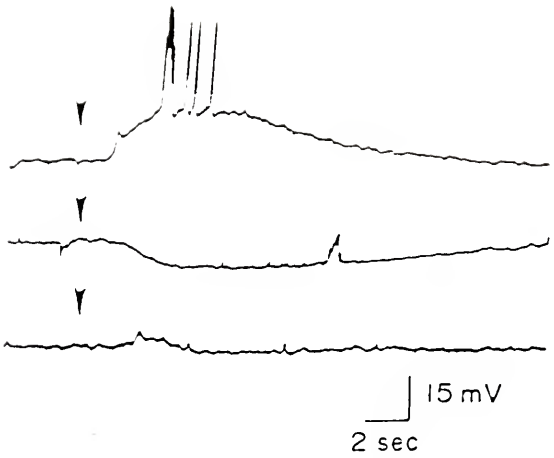

15mV
2 sec

Fig. 5-4. Current-clamp records from an American lobster olfactory receptor cell. Top trace: A large depolarization evoked 1:100 dilution of TET. Membrane potential = -68 mV. Middle trace: A hyperpolarization evoked by 100 μM AAM. Membrane potential = -57 mV. Bottom trace: Injecting a mixture of TET and AAM at the same concentrations used in the first two traces evoked only a small depolarizing response. Membrane potential = -68 mV. Odorants injected at arrow.

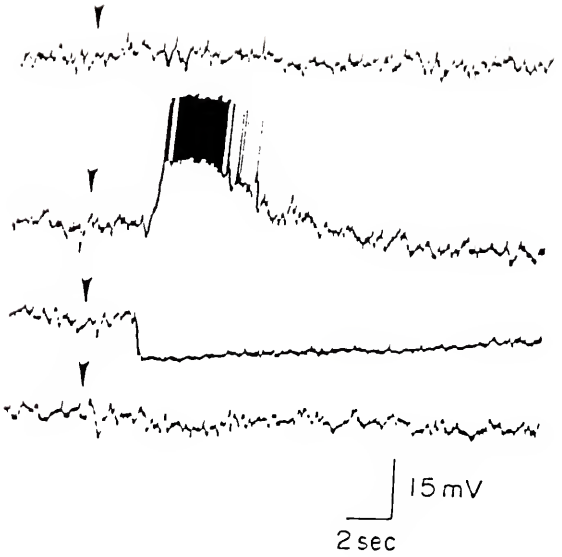

15 mV
2 sec

five somata, all tested at holding potentials of -60, -30 and -10 mV, responded to 0.1 mM AAM applied via pressure ejection pipettes.

Another odorant, histamine, also evoked hyperpolarizing receptor potentials when applied to the dendrites (Fig. 5-5). In H. americanus, histamine caused five cells to hyperpolarize and five cells to depolarize, while nine cells were unaffected. In P. argus, histamine caused two cells to hyperpolarize and four cells to depolarize; two were unaffected. Cells that hyperpolarized to AAM did not necessarily hyperpolarize to histamine. Of the 11 cells hyperpolarized by AAM in both species of lobster, 2 were hyperpolarized by histamine, 3 were depolarized, 3 were unaffected, and 3 were not challenged with histamine. Unlike AAM, histamine is known to gate a chloride channel that occurs on the somata of virtually every olfactory receptor cell in both species of lobster (Chapter 6; Bayer et al., submitted). Although the leak tests were always negative, the assumption that hyperpolarizing receptor potentials in response to histamine are mediated by receptors on the outer dendritic segments must be more tentative than in the case of the amino acid stimuli.

Hyperpolarizing receptor potentials could be produced by increased conductance to ions with highly negative Nernst potentials or to decreases in spontaneously active conductances to ions with Nernst potentials more positive than rest. As an initial attempt to understand the ionic gradients present in lobster olfactory receptor cells, low Cl^- saline (substituting Na gluconate and Na acetate for the NaCl in lobster saline) and high K^+ saline

Fig. 5-5. Current-clamp records from a spiny lobster olfactory receptor cell. Top trace: No response was evoked by injection of a saline blank. Membrane potential = -62 mV. Second trace: A large depolarization and spiking was evoked by 1:100 TET. Membrane potential = -54 mV. Third trace: A hyperpolarization was evoked by 100 μM histamine. Membrane potential = -49 mV. Bottom trace: No response was evoked by 1 μM histamine. Membrane potential = -48 mV. Odorants injected at arrow.

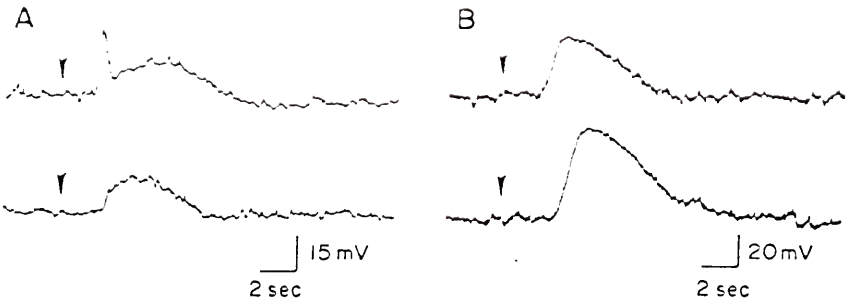

15 mV
2 sec

(substituting 200, 300 or 400 mM KCl for an equal amount of NaCl in lobster saline) were applied to the outer dendritic segments, i.e., as odorants. Both treatments consistently produced depolarizations which mimicked the temporal profile of receptor potentials (Fig. 5-6). In H. americanus, five of six cells were depolarized by dendritic application of a low Cl^- saline and six of six cells were depolarized by high K^+ saline. Similar data were obtained from P. argus; 23 of 30 cells were depolarized by dendritic application of a low Cl^- saline and 30 of 31 cells were depolarized by high K^+ saline. These results indicate that steady-state conductances for both ions exist in the dendrites and that both K^+ and Cl^- have negative Nernst potentials, as commonly found in neurons. These ions, as well as reduction of an inward current, are therefore potential carriers of the outward currents that underlie the hyperpolarizing receptor potentials.

Discussion

Several lines of evidence indicate that the observed odor-evoked hyperpolarizations were not experimentally imposed: (1) they occur in response to biologically-relevant stimuli, (2) they increase in amplitude with increasing stimulus concentration, (3) they originate in the outer dendritic segments, the presumed site of olfactory transduction, (4) they are not expressed in all cells, (5) they are expressed in cells that can also be depolarized by other odorants, and (6) ionic gradients in the outer dendritic segments are physiological and are not perturbed by the patch

Fig. 5-6. Current-clamp records from lobster olfactory receptor cells. A. H. americanus. Top trace: Depolarization evoked by a saline containing 370 mM Na gluconate + 110 Na acetate substituted for the 460 mM NaCl in normal lobster saline. Bottom trace: Depolarization evoked by 300 mM KCl substituted for an equal amount of the NaCl in normal lobster saline. Membrane potential = -61 mV. B. P. argus. Top trace: Depolarization evoked by 480 mM Na acetate substituted for the NaCl in normal lobster saline. Bottom trace: Depolarization evoked by 400 KCl substituted for an equal amount of NaCl in normal lobster saline. Membrane potential = -70 mV. Altered salines injected at arrow.


A
B
15 mV
2 sec
20 mV
2 sec

solution, which would require many minutes to diffuse into the outer dendritic segments.

Hyperpolarizing receptor potentials were not uncommon, at least in the American lobster, where they occurred in 63 % of the olfactory receptor cells challenged with AAM. The small amplitude (relative to odor-evoked depolarizations), stimulus selectivity and coexistence with depolarizing conductances could explain why hyperpolarizing responses to odors have not been recorded previously in lobster olfactory receptor cells and possibly in those of other species, as noted in the Introduction. Finding hyperpolarizing receptor potentials is, perhaps, not surprising. Sensory cells capable of both excitation and inhibition have increased potential for encoding information over purely excitatory systems and even in spontaneously silent cells inhibition can be expressed by reducing net excitation. Natural odors are rarely, if ever, single substances but rather complex mixtures presumably capable of causing excitation and inhibition. Inhibition in olfactory receptor cells would enhance the contrast between patterns of excitation that are thought to be generated across the population of receptor cells by different odorants.

My ability to localize depolarizing and hyperpolarizing receptor potentials to the same cell strongly implies that some lobster olfactory receptor cells possess at least two transduction mechanisms. The two mechanisms presumably differ at least in either the species of ionic conductance altered or the biochemical mechanism by which a single conductance is altered. The alternative that the outer dendritic segments of any one cell are

compartmentalized, with some compartments having inverted ionic gradients, and that the compartments are selectively stimulated by certain odorants, does not seem likely from general principles of neuronal organization. In fact, the consistency of responses to transient changes in the extracellular K^+ and Cl^- concentrations bathing the dendritic segments suggests that such compartmentalized ionic gradients do not occur. More difficult to dismiss is the alternative that a single second messenger system derives ongoing activity from a spontaneously active enzyme linked directly to receptor proteins, i.e., a GTP-binding protein, that in turn may be enhanced and suppressed by the receptor proteins. No precedent exists, however, for GTP-binding proteins to be inhibited by interaction with receptor proteins (Gilman, 1987). Bidirectional regulation of second messenger production by distinct GTP-binding proteins, as occurs with G_s and G_i acting on adenylate cyclase (Bokoch et al., 1984), represents two distinct transduction mechanisms in my view. My proposal of multiple transduction mechanisms in lobster olfactory receptor cells is in agreement with evidence for both cAMP-dependent transduction (Pace et al., 1985; Sklar et al., 1986; Nakamura and Gold, 1987) and an odorant-gated channel (Labarca et al., 1988) in amphibian olfactory receptor cells and with evidence for multiple mechanisms for transduction in another chemosensor, vertebrate taste cells (reviewed by Kinnamon, 1988).

A second implication of hyperpolarizing and depolarizing responses occurring in the same cell is that it provides a

functional, noncompetitive mechanism for peripheral mixture suppression in the lobster olfactory pathway. Mixture suppression is the reduction in the intensity of response to a stimulatory compound when presented in mixture (e.g., Bartoshuk, 1975). In this instance, suppressive odor components would either shunt depolarizing inward currents by eliciting opposing, outward currents or suppressive and stimulatory odor components would activate opposing intracellular biochemical interactions. Towards this end, I found that simultaneous presentation of inhibitory odorants (AAM) with excitatory ones (TET) reduces the magnitude of the excitation. Previous extracellular studies have shown that proline, arginine, and cysteine, the constituents of AAM, suppress the output of spiny lobster olfactory receptor cells, and suggested that they do so both by competing for common receptor sites and by an additional, noncompetitive mechanism (Ache, Gleeson and Thompson, 1988). The presence of opposing odor-evoked currents or opposing intracellular biochemical processes, as suggested by my results, would provide the latter mechanism. As noted by Getchell (1986), however, mechanisms associated with suppression of olfactory receptor cell activity are ill-defined relative to emerging concepts regarding mechanisms associated with excitation. These findings may provide the impetus to begin searching for those mechanisms.

CHAPTER 6
A LIGAND-GATED CHANNEL RECEPTOR FOR HISTAMINE

Introduction

In several sensory modalities, modulation of the sensitivity of primary receptor cells by centrifugal synaptic inputs or by hormones seems to be an important mechanism for adapting sensory input to match environmental or internal physiological conditions (Kass and Renninger, 1988; Pasztor and Bush, 1987; Art et al., 1982; Flock and Russel, 1973; Hagiwara et al., 1960). Indirect evidence from frogs (Bouvet et al., 1987) and mosquitoes (Davis and Takahashi, 1980) suggests that such modulation may also occur in olfaction. The finding that histamine (HA) suppresses the excitability of lobster olfactory receptor cells substantiated and extended these observations (Bayer et al., submitted). Application of HA to the somata of lobster olfactory receptor neurons suppresses both spontaneous and odor-evoked spiking and reduces the amplitude of receptor potentials. Only HA is capable of producing these effects; other biogenic amines, HA analogs, and transmitters such as Γ-aminobutyric acid (GABA) and acetylcholine are ineffective. The pharmacology of the receptor mediating this effect is similar to the pharmacology of a fast HAergic synapses in insects (Hardie, 1987; 1988).

HA is a putative neurotransmitter and important neuroregulatory compound in diverse species (McCaman and Weinrich, 1985; Prell and Green, 1986; Schwarz et al., 1986). HA responses investigated to date appear to be mediated by intracellular biochemical second messengers (Sasaki and Sato, 1987; Vogel et al., 1987; Brown et al., 1984; Daum et al., 1984; Hegstrand et al., 1976). Recent evidence that HA mediates fast synaptic transmission in arthropods (Claiborne and Selverston, 1984; Hardie, 1987, 1988; Simmons and Hardie, 1988; Laughlin et al., 1987) and molluscs (McCaman and Weinrich, 1985; Gruol and Weinrich, 1979) raises the possibility of a more direct action of HA, however. Herein, I provide evidence that lobster olfactory receptor cells express a chloride channel directly gated by HA. Evidence is also provided to support the conclusion that this channel mediates the suppressive action of HA previously observed in these cells (Bayer et al., submitted).

Methods

Adult specimens of P. argus were collected in the Florida Keys and maintained in flowing seawater at 18 to 23°C. Similar specimens of H. americanus were purchased from commercial suppliers in Woods Hole, MA and maintained in flowing seawater at 13 to 17°C. Olfactory receptor cells were prepared for voltage-clamp and single channel recording as described in Chapter 2 and Chapter 3, respectively. Somata were isolated and dispersed into a recording dish using a single trituration step. Outside-out patches were

usually excised from somata which remained attached to clusters of olfactory receptor cells.

HA was applied by pressure ejection from glass pipettes with 1 μm tip diameters or, when steady-state concentrations were desired, by placing outside-out patches into a gravity-fed flow from a large bore pipette connected to six solution reservoirs via a six-way valve. Each antagonist was pre-mixed with HA in saline and applied via separate pressure ejection pipettes. Intracellular perfusion of drugs dissolved in a patch solution was accomplished by diffusion of the patch solution into the cell during whole-cell recording. Nernst potentials were calculated using ion concentrations corrected for activity coefficients.

The composition of lobster saline was (mM) 460 NaCl, 13 KCl, 13 $CaCl_2$, 10 $MgCl_2$, 1.7 glucose, 10 HEPES, pH 7.4. The composition of choline Cl saline was (mM) 450 choline Cl, 50 NaCl, 20 $MgCl_2$, 0.01 HA, 20 HEPES, pH 7.4. The composition of NaCl saline was (mM) 500 NaCl, 10 $MgCl_2$, 20 HEPES, pH 7.4. The composition of the normal patch solution was (mM) 200 KCl, 11 EGTA, 1 $CaCl_2$, 2 $MgCl_2$, 570 glucose, 10 HEPES, pH 7.0. The composition of the low Cl^- patch solution was (mM) 26 KCl, 180 K acetate, 2 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, 570 glucose, 10 HEPES, 34 NaOH, pH 7.0. The composition of NaCl patch solution was (mM) 160 NaCl, 20 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 570 glucose, 10 HEPES, pH 7.0. The composition of Na gluconate patch solution was (mM) 200 Na gluconate, 13 K acetate, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 560 glucose, 10 HEPES, pH 7.0. Inorganic salts were purchased from Fisher Scientific (Fair Lawn, NJ) and HEPES and EGTA

were purchased from Research Organics (Cleveland, OH). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Applying HA to isolated, voltage-clamped somata of lobster olfactory receptor cells evoked a macroscopic current associated with an increase in membrane conductance (Fig. 6-1A). The current reversed close to the Nernst potential for Cl^- (E_{Cl}) and shifted with changes in E_{Cl} (Fig. 6-1B). The slight positive shift in the reversal potential relative to E_{Cl} presumably reflects the small sodium permeability of the underlying channel (see below). Neither intracellular perfusion of 0.2 or 1 mM GTP-Γ-S (N = 16), a GTP-binding protein activator, nor 1 mM guanosine 5'-O-(2-thiodiphosphate) (N = 3), a GTP-binding protein inhibitor, affected the ability of HA to activate the macroscopic current in voltage-clamped somata. This suggests that the HA response was not mediated by a GTP-binding protein. Adding 5 mM Co^{++} and 5 mM Cd^{++}, potent calcium channel blockers, to the external saline also was without effect on the macroscopic current (N = 2), suggesting that Ca^{++} influx was not required. No channel activation was observed in cell-attached patches on isolated somata when HA was applied outside the patch pipette (N = 19), even though spiking was suppressed in the two somata which spiked spontaneously. This result obviates mechanisms acting by cytoplasmic second messengers. HA applied to the external face of outside-out patches, however, frequently resulted in activation of a chloride channel (N = 36; Fig. 6-2A). The requirement for external HA for gating of this channel in

Fig. 6-1. Macroscopic currents from isolated somata of H. americanus olfactory receptor cells. A. An inward current accompanied by a conductance increase evoked by a 0.2 s pulse of 10 μM HA applied (arrow) to a soma held at -70 mV. 30 mV voltage pulses were injected to monitor conductance. Pipette: normal patch solution. Bath: saline. B. Currents from two other somata activated by 0.1 s pulses of 100 μM HA applied (arrows) to somata held at the voltages shown. Currents from a soma perfused to establish E_{Cl} at -23 mV (left panel; pipette: high Cl^- patch solution) reversed at a less negative potential than those from another soma perfused to establish E_{Cl} at -63 mV (right panel; pipette: low Cl^- patch solution). Bath: saline.

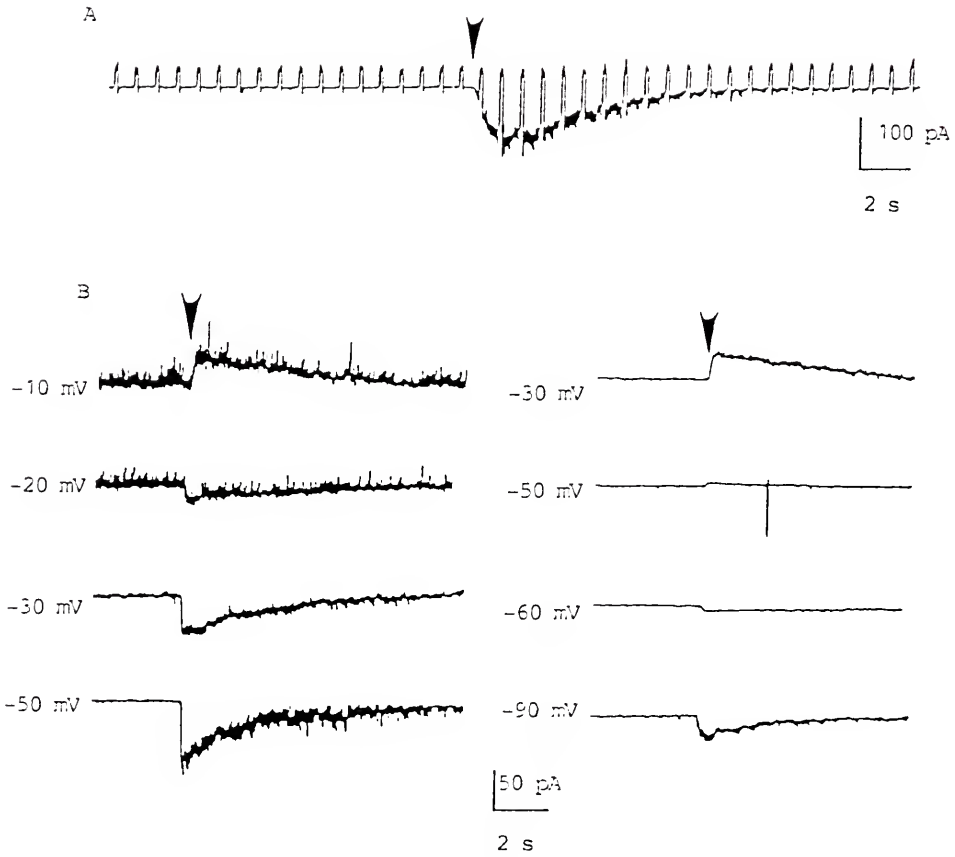
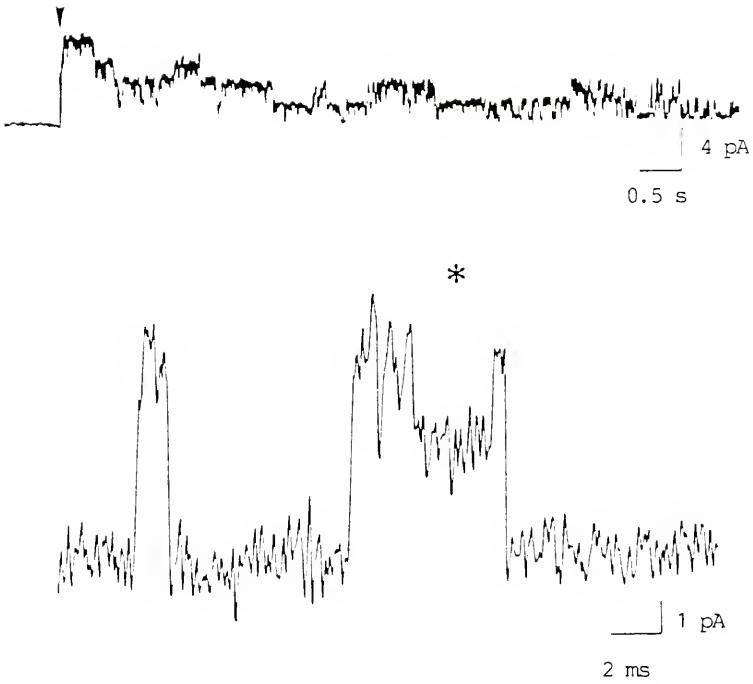

A
100 pA
2 s
B
-10 mV
-20 mV
-30 mV
-50 mV
-30 mV
-50 mV
-60 mV
-90 mV
50 pA
2 s

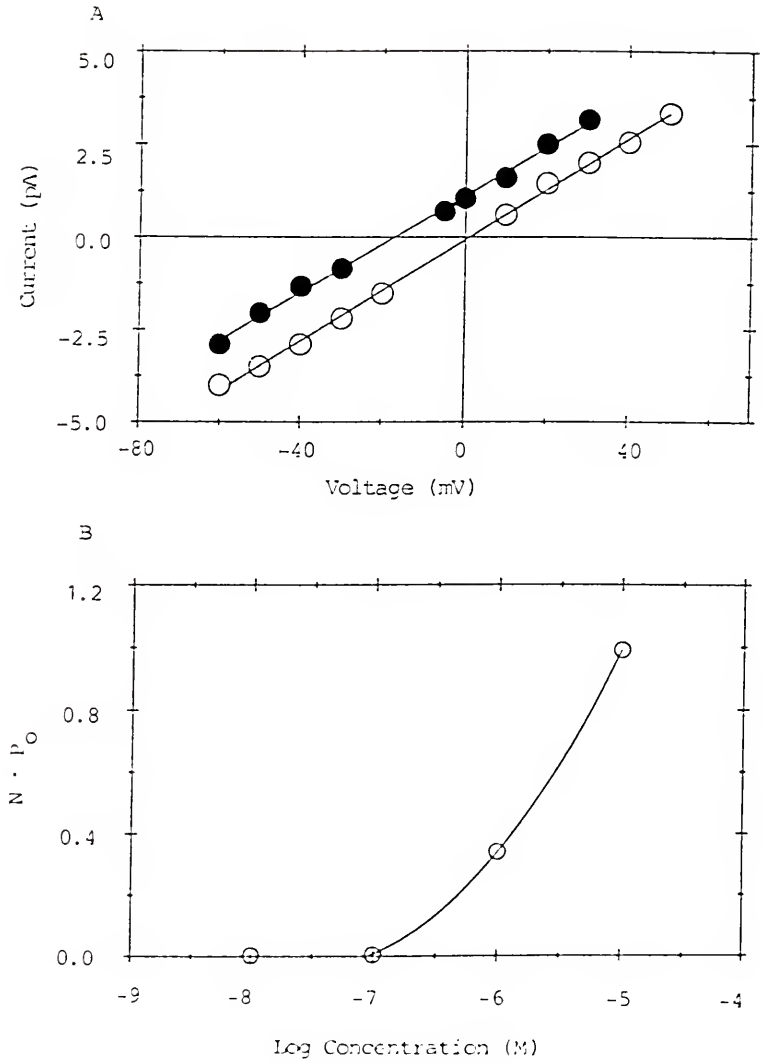
Fig. 6-2. Unitary current events from excised patches of lobster olfactory receptor cell somata. A. Four channels activated by a 0.4 s pulse of 100 μM HA applied (arrow) to an outside-out patch from a H. americanus olfactory receptor cell. Openings are upward. Pipette: low Cl^- patch solution. Bath: saline. Patch held at 0 mV. B. An opening which transiently reverted to a subconductance state (*) in an inside-out patch from P. argus. Pipette: choline Cl saline. Bath: NaCl saline. Patch held at 50 mV.


4 pA
0.5 s
*
1 pA
2 ms

outside-out patches was not affected by adding 1 mM GTP-Γ-S to the cytoplasmic face of the patch (N = 3), eliminating the alternative that a membrane-diffusible GTP-binding protein activates the channel (Codina et al., 1987; Yatani et al., 1987; Logothetis et al., 1987) Including HA in the patch pipette allowed recording of this channel in cell-attached and inside-out patches (N = 6). HA-dependent gating of the channel in outside-out and inside-out patches proceeded without noticeable decreases in activity for the duration of seal retention (up to 30 min) in the absence of nucleotide triphosphates, evidence that gating did not require an energy dependent biochemical reaction. Taken together, these results demonstrate that HA acted by directly gating a channel.

The HA receptor channel had mean slope conductances of 44 ± 6 pS in H. americanus and 66 ± 6 pS in P. argus (Fig. 6-3A). Subconductance states occurred (Fig. 6-2B), but their rarity precluded accurately estimating their slope conductance. Dose-response measurements based on the percentage of time the channel was in the open state showed that activation of the channel occurred at steady-state HA concentrations of 1 μM or greater (Fig. 6-3B; N = 2). Kinetic analysis indicated at least two exponential components to both the open and closed duration distributions. The mean time constants of the open durations were 1.4 ± 0.2 ms and 8.4 ± 1.1 ms, and the mean time constants of the closed durations were 1.1 ± 0.1 ms and 27.3 ± 14.6 ms for 4 channels held between 10 and 30 mV. The channel tended to spend more time in the open state at depolarized potentials. For two channels in inside-out patches

Fig. 6-3. Plots of the ion selectivity and dose-dependency of the HA receptor channel. A. Plot of the channel amplitude versus holding potential for two channels from P. argus olfactory receptor cells under conditions with different Nernst potentials for Cl^-. Open circles: A channel in an inside-out patch bathed in symmetrical choline Cl saline (E_{Cl} = 0 mV) with 10 μM HA in the pipette reversed at 1.2 mV. Closed circles: Another channel in an outside-out patch under ionic conditions where E_{Cl} = -23 mV reversed at -17.3 mV. A Na:Cl permeability of 0.15 was calculated for this channel. Pipette: NaCl patch solution. Bath: NaCl saline. B. Plot of the percent of time spent in the open state (P_o) times the number of channels in the patch (N) versus the log of the steady-state HA concentration. Data from an outside-out patch containing 4 channels from H. americanus. Pipette: Na gluconate patch solution. Bath: saline. Patch held at 23 mV.


A
5.0
2.5
0.0
-2.5
-5.0
Current (pA)
-80
-40
0
40
Voltage (mV)
B
1.2
0.8
0.4
0.0
N · P_O
-9
-8
-7
-6
-5
-4
Log Concentration (M)

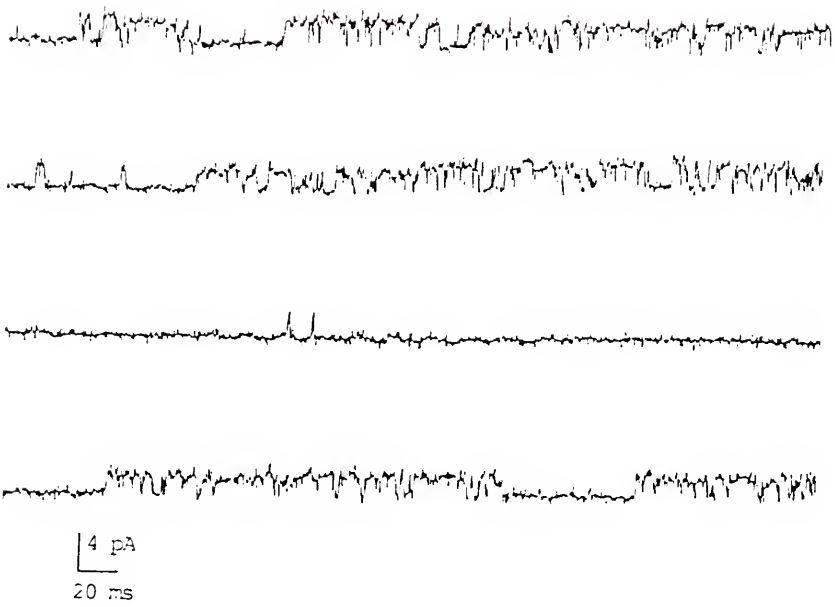
recorded with pipettes containing 10 μM HA, the percent of time spent in the open state increased 9.5- and 16.8-fold from -50 to 50 mV. The channel was also permeable to at least one cation. The permeability ratio of Na:Cl was calculated from the Goldman-Hodgkin-Katz equation using reversal potentials which were measured with Na^+, Cl^-, and Mg^{++} (necessary for seal formation and stability) as the only potentially permeant ions (Fig. 6-3A, closed circles). Mg^{++} was ignored in the calculation because shifting its Nernst potential did not affect the reversal potential (compare Fig. 6-1b and Fig. 6-3A). Na:Cl permeability ratios calculated for two channels were 0.15 and 0.13.

The pharmacology of the channel agreed with that obtained from extracellular recordings of HA's suppression of spiking (Bayer et al., submitted). Cimetidine, a vertebrate H_2 histamine receptor antagonist, blocked the ability of HA to activate the channel while pyrilamine, an H_1 antagonist, was without effect (Fig. 6-4; N = 3).

Discussion

The lack of effect of GTP analogs, the lack of requirement for nucleotide triphosphates or other energy sources, and the absence of effect of Ca^{++}, which was applied at 0.1 μM to 13 mM to the intracellular side of the channel in inside-out versus outside-out patches, is inconsistent with the involvement of any known second messenger system in gating the HA channel. All my evidence is consistent with the conclusion that the HA receptor is a ligand-gated channel similar to a nicotinic acetylcholine receptor or a $GABA_A$ receptor. This finding is in direct contrast to HA effects

Fig. 6-4 Unitary current events from an outside-out patch from an olfactory receptor cell of H. americanus. Openings activated by a 0.5 s pulse of 10 μM HA (top trace) were unaffected by 500 μM pyralimine (second trace), but were virtually abolished by 500 μM cimetidine (third trace). The effect of cimetidine was reversible on allowing time for diffusion to reduce its concentration (bottom trace). Openings are upward. Pipette: low Cl^- patch solution. Bath: saline. Patch held at 10 mV.


4 pA
20 ms

mediated by second messengers in molluscan neurons (Sasaki and Sato, 1987; Vogel et al., 1987) and to biochemical evidence from mammalian brain linking HA receptors to second messengers (Prell and Green, 1986; Schwartz et al., 1986; Brown et al., 1984; Daum et al., 1984; Hegstrand et al., 1976). Many of the properties of the HA receptor; chloride permeability, voltage dependence, subconductance states, bursting behavior, ohmic behavior, and slope conductances, are similar to the $GABA_A$ and glycine receptors (Huck and Lux, 1987; Bormann and Clapham, 1985; Weiss, 1988; Blair et al., 1988). An important difference, however, is the small Na^+ permeability of the HA receptor channel (Bormann, 1986). Cation permeability is not uncommon among some other anion channels, however (Franciolini and Nonner, 1987; Coulombe et al., 1987; Stanley et al., 1988). The pharmacology and the exact match of the dose dependency of channel activation and spike suppression (Bayer et al., submitted) suggest that the HA receptor channel underlies the suppression of spiking. Previous evidence from recordings of steady-state chloride channels which are common in the somata of the lobster olfactory receptor cell (Chapter 3) indicates that E_{Cl} is near the resting membrane potential in these cells. Activation of a Cl^- conductance by HA would effectively clamp the membrane potential near rest, thereby explaining the suppression of spiking and the shunting of receptor potentials. The similarity of the pharmacology of the HA receptor to that of postsynaptic HA responses in insect visual neuropil (Hardie, 1987, 1988; Simmons and Hardie, 1988; Laughlin et al., 1987), which are mediated by an increase in Cl^- conductance (Zettler

and Straka, 1987), suggests that a similar receptor may underlie inhibitory responses to HA in other organisms.

The ubiquity of the HA receptor on receptor cell somata contrasts with the relative paucity of responses to HA applied to the dendrites as an odorant (Bayer et al., submitted). Furthermore, most of these dendritic responses are excitatory. These observations indicate that the function of the somatic HA receptor is not to mediate odorant transduction. Immunohistochemical and biochemical experiments detected HA and HA synthesis, respectively, in regions of the lobster central nervous system where the receptor cell axons terminate, but not in the peripheral olfactory organ (Orona, Bayer, Grieco, Battelle and Ache, unpublished data) where there is no evidence of centrifugal or collateral synapses (Grünert and Ache, 1988; Spencer, 1986; Spencer and Linberg, 1986). If the HA receptor, like other neurotransmitter-gated channels, is a synaptic receptor, then the terminals of the receptor cell axons may be a target of histaminergic input. HA is known to mediate presynaptic inhibition, an important form of synaptic plasticity, in both vertebrates (Tamura et al., 1988) and invertebrates (Kretz et al., 1987). The implication that somatic HA receptors are strays from distant synapses is not without precedent among other biogenic amine receptors in invertebrates (Siegelbaum et al., 1986). Regardless of their functional location, the expression of this receptor in olfactory receptor neurons implies that these neurons are the target of some as yet undescribed regulatory or feedback process.

CHAPTER 7
SUMMARY

These studies describe many of the ion channels and currents which underlie the electrical properties of the olfactory receptor cell of P. argus. Barring the unlikely occurrence of extremely rapid washout of other currents, the four voltage-dependent ionic currents found; a Ca^{++} current, a Na^{+} current, a delayed rectifier K^{+} current, and a Ca^{++}-activated K^{+} current, are the full complement of currents in the soma and the axon. Because these ionic currents could contribute little to the spread of inward currents at potentials below threshold (-45 to -30 mV; Schmiedel-Jakob et al., 1989), odor-evoked currents must spread passively through the soma to the axon where spikes are generated in lobster olfactory receptor cells (Schmiedel-Jakob et al., 1989), as appears to be true in their vertebrate counterparts (Firestein and Werblin, 1987; Trotier, 1986). The linearity or near linearity of odor dose-response functions observed in extracellular recordings is remarkable (Derby et al., 1984; Gleeson and Ache, 1985) when one considers that the inward Ca^{2+} current adds to the odor-evoked current while the outward K^{+} currents act to shunt it away. The balance achieved between these currents and the odor-evoked inward current is an intriguing question for future study. A related question of equal importance is how the K^{+} currents participate in determining the

interspike interval that determines the frequencies of spike trains (Connor, 1976). These spike trains presumably encode the information passed on to the CNS. The properties of the ion channels described, though certainly not inclusive of every ion channel type present in the somata of the olfactory receptor cell, further supports the conclusion that odor-evoked currents spread passively. The voltage-independent Cl^- channel may be a significant factor in stabilizing the resting membrane potential of the olfactory receptor cell. This conclusion is consistent with anecdotal evidence about instabilities produced in the membrane potential when the external Cl^- concentration is reduced during intracellular recording (Schmiedel-Jakob, personal communication). The nonselective cation channel is apparently not active in mature neurons under physiological ionic conditions and its unknown functions therefore do not appear to include participation in receptor cell activation.

These studies also describe the results of tests of some specific hypotheses. I tested the hypothesis that responses to the complex odor mixture, TetraMarin extract, were mediated by the production of cAMP, and found little evidence to support this hypothesis. TetraMarin extract stimulates such a large percentage of the receptor cells tested (McClintock, personal observation) that these results suggest that transduction mechanisms other than cyclic nucleotides are linked to most odor responses in lobster olfactory receptor cells. Other potential mechanisms include ligand-gated channels such as those observed in other chemosensory cells (Labarca

et al., 1988; Hatt and Francke, 1987), phosphoinositide metabolism as in responses to amino acid odorants in catfish olfactory cilia (Huque and Bruch, 1986), and direct activation of ion channels by G-proteins as in mammalian cardiac myocytes (Codina et al., 1987; Yatani et al., 1987). These mechanisms remain to be tested in lobster olfactory receptor cells.

I also found evidence in support of the hypothesis that the suppressive components of an odor mixture (Derby and Ache, 1984a) could evoke hyperpolarizing receptor potentials. Simultaneous application of depolarizing and hyperpolarizing odorants caused the reduction or abolition of the depolarizing receptor potential. This suggests that at least one mechanism of mixture suppression, probably corresponding to suppressive effects which are not consistent with receptor-site competition (Ache et al., 1988), is the shunting of an odor-evoked inward current through an outward current activated simultaneously. These data also provide circumstantial evidence to support the proposal that multiple transduction mechanisms exist in lobster olfactory receptor cells (Ache et al., 1989) and suggests that these multiple mechanisms may coexist in the same cell.

The hypothesis that HA suppressed the excitability of olfactory receptor cells by directly gating a channel was also supported by the results of my experiments. This HA receptor has interesting similarities to the $GABA_A$ receptor, including being a Cl^- channel and being sensitive to voltage (Blair et al., 1988; Weiss, 1988). Such ligand-gated channel receptors are almost exclusively found at

synapses where they mediate rapid responses. With the exception of those which pass Ca^{2+} (MacDermott et al., 1986), such receptors can only cause effects for the duration of the presence of the ligand. Although local release in the antennule, such as from a neurohemal organ, cannot be excluded, evidence which includes morphology (Grünert and Ache, 1988; Spencer, 1986) and localization of HA and HA synthesis (Orona, Bayer, Grieco, Battelle and Ache, unpublished data) suggests that the location of HA's action on the lobster olfactory receptor cell may be at their axon terminals in the glomerular regions of the CNS. The partial pharmacological characterization of the HA receptor (Bayer et al., submitted; Chapter 6) provides information which can be used to test this hypothesis in physiological experiments using recording from the olfactory regions of the CNS.

These studies have not only provided information about some of the properties of the olfactory receptor cell, but have also contributed to a speculative view of olfactory receptor cell function which can guide further research. Odorants access the outer dendritic segments when the lobster samples its environment by "flicking" its antennules (Schmitt and Ache, 1979). These odorants will be cleared by degradative enzymes, by uptake (Trapido-Rosenthal et al., 1987; Gleeson et al., 1987) and by the next flick. Before being cleared, the odorant molecules presumably activate receptor proteins on the outer dendritic segment. The activated receptors must then initiate transduction in order to generate the initial electrical signal. Evidence from Chapter 5 suggests that several

mechanisms may be involved, perhaps even for the same odorant because the suppressive mixture of L-proline, L-arginine and L-cysteine can evoke both excitatory and inhibitory responses. Activation of adenylate cyclase does not appear to mediate at least the majority of odor responses. Conductance and reversal potential measurements suggest that odor-evoked electrical responses are generated by either increases or decreases in conductance, and may be carried by more than one ion species permeating a single nonselective channel or several, more selective channels (Ache et al., 1989). The initial electrical signals generated within some receptor cells sometimes interact, i.e. hyperpolarizing and depolarizing responses, and generate unique responses which presumably enhance the breadth of coding in individual receptor cells. Odor-evoked depolarizations reaching the soma, apparently by passive spread, activate the voltage-dependent currents which must generate and shape the burst of spikes which carry information to the CNS. Once threshold is reached, outward rectification provided by the K^+ currents decreases the magnitude of change of membrane potential caused by inward currents. This allows a wider range of odor concentrations to cause depolarizations that fall within the working range of the cell, the membrane potentials evoking the minimum (threshold) to the maximum spike frequencies. The Ca^{2+}-activated K^+ current may help to determine spike frequency, as in some other neurons (Barrett and Barrett, 1976). Because TTX eliminates spiking in these cells (Schmiedel-Jakob et al., 1989), the Na^+ current must underlie the action potentials. The evidence

that the Na^+ current may be concentrated in the axon is consistent with evidence from intracellular recordings that spikes are generated there (Schmiedel-Jakob et al., 1989). These spikes propagate to the CNS, presumably carrying encoded information about the odorant. This information may not reach the CNS independent of input from other processes, however, because of the evidence that feedback or regulatory processes may affect the cell via release of HA (Bayer et al., submitted). The ligand-gated nature of the HA receptor suggests histaminergic synapses, or perhaps local release, as sources of HA acting on the lobster olfactory receptor cell.

The lobster olfactory receptor cell has been a leading model of olfactory receptor cell function. Because the means are available to test the speculations proffered in this view of its function, the lobster olfactory receptor cell should continue to be an important model for studies of the physiology and biochemistry of primary olfactory neurons.

APPENDIX
A SIMPLIFIED EXPLANATION OF SINGLE CHANNEL RECORDING

This appendix is a practical discussion of how single channel recordings are made, what kinds of information can be obtained from such recordings, and consideration of some advanced applications pertaining to research presented in Chapter 3. For a brief, but excellent treatment of the present state of understanding of ion channels I recommend Miller (1987). More detailed discussions can be found in Moczydlowski (1986), Sakmann and Neher (1983) and Hille (1984).

The critical discovery which made single channel recording possible was that glass and lipid bilayers could be made to physically "seal" to each other (see Hamill et al., 1981). This seal is electrically tight, meaning that the apposition of glass and membrane is so close that few ions can leak out between them. The seal is also so physically strong that the cell membrane will usually break before the seal when increasing stress is applied in the form of suction or movement of the glass pipette. Glass pipettes made with a hole of about 1 μm in diameter are normally used to form seals, often with the aid of gentle suction applied to the lumen of the pipette. The membrane under the pipette may be left undisturbed, termed "cell-attached" or the whole piece of membrane sealed to the pipette may be excised from the cell, termed an "inside-out" patch because the inner surface of the membrane now

faces the bath. Alternatively, the membrane under the seal may be ruptured by a second, stronger suction, creating the "whole-cell" configuration, which allows simultaneous recording from all the ion channels in the cell, analogous to more classical methods of voltage-clamp recording (e.g. Hodgkin and Huxley, 1952). If the pipette is then withdrawn from the cell, a neck of membrane is pulled out which eventually breaks and immediately seals over on the tip of the pipette, termed an "outside-out" patch because now the external surface of the cell membrane is facing the bath.

The insulative properties of the glass and the lipid bilayer, and the high electrical resistance of the seal allow currents of about 0.5 pA or larger to be measured. The thermal and conductive properties of the recording electronics and the glass create current fluctuations or "noise" which set the lower limit of detectable current. An operational amplifier and feedback resistor are used to voltage-clamp the patch of membrane to any desired voltage. Currents flowing across the membrane are immediately inverted and injected back across the membrane by the feedback resistor and operational amplifier circuit to maintain the voltage-clamp. This injected current is measured across a series resistor from the bath to the circuit ground.

The current flowing through an open ion channel appears as an instantaneous jump in current, which returns to its original level just as rapidly when the channel closes. In a patch with only one channel, this creates a current record with fluctuations between two current levels. Opening and closing of ion channels is believed to

be due to conformational change of the channel protein masking and unmasking a pore. The closed and open current levels can be distinguished by two methods. (1) At a holding potential where the channel is either inactive or too small to detect, the seal resistance (R_s) is measured by injecting known voltage steps of small amplitude and measuring the current offset, which is the current flowing across the seal (I_s). At any holding potential (V), the current level which matches the current across the seal resistance ($I_s = V/R_s$) is therefore the closed level. (2) The electrical background noise, i.e. the width of the current trace, is measured as peak-to-peak noise, for example. Because this background noise is increased when current flows, the open current level will be the wider trace.

There are obviously few things that can be measured in a recording which only fluctuates between two levels. The amplitude of the open current level can be used to construct a current-voltage (I-V) plot if it is measured at several voltages (e.g. Fig. 3-1B). The slope of this plot gives the conductance of the channel. The voltage where the current is zero can be compared to the Nernst potentials of the ions present to determine the ion selectivity of the channel. The times spent in the open and closed states can also be measured. The sum of the open times divided by the duration of the recording is often referred to as the probability of opening, or the probability of being in the open state (P_o). The P_o is a convenient measure of the degree of activity expressed by the channel. The distributions of the durations of the openings and

closings can also provide useful information. Compiled as a frequency of events per time versus time histogram (e.g. Fig. 3-4), the distribution describes an exponential function which may have one or more components. The mean duration of either the closed or the open state is easily obtained because it is the sum of the time constants of the exponential components of the distribution. The presence of multiple exponential components in these distributions is most parsimoniously explained by conformational substates of the channel protein, but confirmation of this assertion lies far in the future of the study of protein structure. In any case, the mean open and closed durations are convenient and sensitive measures of the dynamic activity of the channel.

Why are the distributions of open and closed durations exponential? The explanation has its basis in the fact that openings and closings of a channel are the result of events which occur on a scale where random thermal energy changes govern molecular behavior. If one were able to measure the rate at which a large number of channels, all either open or closed, changed to the other state once, one would arrive at an exponential function just as if one had measured the rate of molecular decay of a radioactive sample or the rate of disappearance of a substrate in an enzymatic reaction. When measuring a single channel's gating (opening and closing), one is able to arrive at the same result by repeatedly measuring the times that channel spends in one state before exiting to the other, and constructing a frequency histogram to describe the exponential distribution. Details of the theories underlying

kinetic analysis of channel activity and derivations of the equations involved can be found in Colquhoun and Hawkes (1983) and Moczydlowski (1986).

A related topic involving random molecular processes is the response of channels to stimuli. Although the two state nature of a single channel recording gives the appearance of an all-or-non process, stimulating ion channel gating is not a threshold phenomenon, but rather governed by statistical probabilities. While discussing a simple scheme of gating in terms of the free energy state of the channel molecule, Miller (1987, pp. 51-52) writes,

> "Underlying such a scheme is molecular chaos, and the advent of single channel recording techniques has allowed us to observe this chaos directly. When we say that some external variable 'causes' a channel to open, we really mean, and really observe at the single channel level, that application of this variable biases the statistics of opening - that is, it makes the free energy of the open state relatively more favorable with respect to the closed state."

Changes in membrane voltage, the binding of ligands, covalent modification by enzymes; all act by altering the probability of conformational change in the channel protein.

In addition to its gating properties, the other characteristic feature of any ion channel is the types of ions which pass through its pore. The properties of the channel's pore, more properly of the amino acid chains making up the wall of the pore, determine

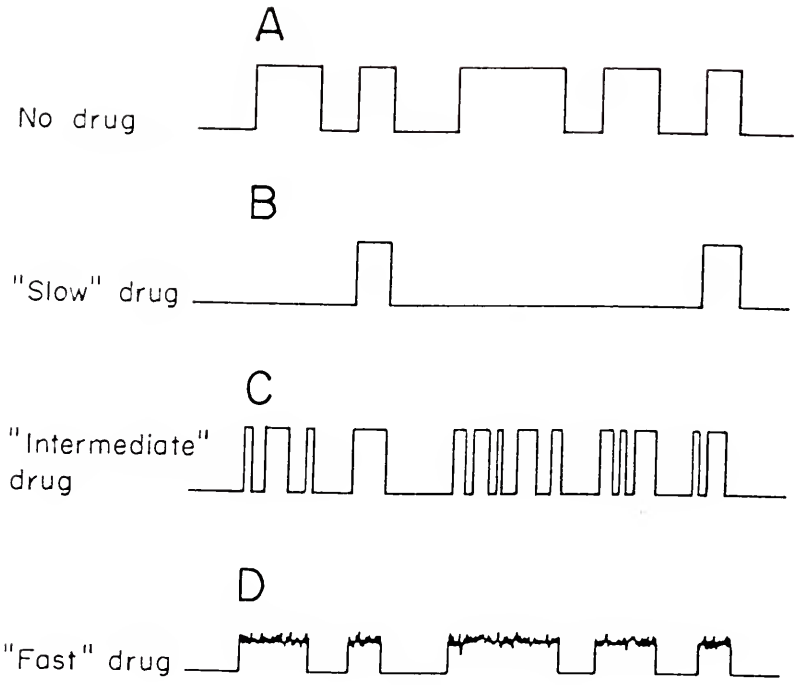
which ions will pass (selectivity) and how fast they will go through (conductance). Ion channels select one ion over another on the basis of physical size, ion charge, and the hydration (solvation) energy of the ion (Hille, 1984). Ion channel pores are so narrow that ions entering it are no longer in a truly aqueous phase but rather encounter forces generated by the pore wall. Waters of hydration are stripped from the ion as it enters the pore and the energy lost in dehydration must be supplied by "binding-sites" in the pore. The ability of these pore binding sites to "solvate" various ions determines how well each ion will permeate the pore (Hille, 1984). Compared to most binding interactions measured in biology, the binding of ions to sites within ion channel pores must be very weak in order to permit the high rate of ion flux observed through ion channels. A consequence of this fact is that an ion which binds more strongly than another ion will permeate more slowly and have a lower conductance.

The last topic to be considered here is block of ion channels. One method of studying the pore of an ion channel is to apply blocking agents. Ion channels can be blocked in two ways, by allosteric interactions which reduce the probability of conformational change to the open state, and, more commonly, by plugging the pore (pore blockers). Pore block can result in several changes in the appearance of single channel recordings. Pore blockers which bind and unbind very slowly prevent openings from being observed as often as normal without changing the channel amplitude (Fig. A-1B). Pore blockers with intermediate kinetics

cause the mean open duration to decrease because discrete closings (blocking events) interrupt the normal open durations (Fig. A-1C). Again, the channel amplitude is unaffected. Pore blockers with fast kinetics reduce the apparent channel amplitude (Fig. A-1D) because the blocking and unblocking of the pore are too rapid to be resolved due to the limited bandwidth of recording amplifiers (Hille, 1984). This "fast block" was observed in Chapter 3 (Fig. 3-5). Block can provide information about the length of the narrow part of the pore, and about the number and location of binding sites within the channel pore. The concentration dependence of block can be used to calculate a Hill coefficient, an estimate of the number of blocking particles involved. If ions or charged molecules are used as blockers, block often proves to be voltage-dependent because the blocker binding sites are often in the part of the pore within the membrane's electric field. Especially in the case of block by inorganic ions, the blocking sites may be the ion binding sites in the pore discussed above. Woodhull (1973) developed equations allowing the calculation of how far into the membrane's electric field a blocker binding site lies. Block by a charged particle can often be relieved by increasing the permeant ion concentration on the side of the membrane opposite to the blocking ion. This "knock-off" effect is proof of multiple ion binding sites within the channel pore if the effect is on the dissociation rate of the blocker. Study of the channel pore using blocking agents is very useful because it is the only available means to generate structural information about physiologically active channel proteins. As

molecular biology and the crystallographic study of integral membrane proteins improve, this information will aid our ability to properly reconstruct the physiological conformations of ion channel proteins.

Fig. A-1. Types of blocking effects which may be observed in single channel recordings depend on the rates of association and dissociation of the blocking molecule. A. A hypothetical stretch of recording with one active ion channel. B. A blocker which dissociates slowly would cause this record to show fewer openings. C. A blocker which associates and dissociates with intermediate kinetics would cause this record to show many more closed events. D. A blocker with rapid kinetics would cause the apparent open channel amplitude to decrease. After Hille (1984).


A
No drug
B
"Slow" drug
C
"Intermediate"
drug
D
"Fast" drug

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BIOGRAPHICAL SKETCH

Timothy S. McClintock was born September 30, 1959 in Gallipolis, Ohio. He resided for a short time in West Virginia before moving on to two small communities in Ohio, graduating in 1978 from Spencerville High School in Spencerville, Ohio. During this period he acquired an interest in biology from his father, a science teacher. He matriculated at Michigan State University to study zoology, graduating with high honors in June, 1982. A stint at the Bermuda Biological Station in 1981 led to his desire to study the behavior of marine organisms. He went to Florida State University, earning a Master of Science degree in biological sciences in 1984. With his interests now firmly rooted in the sensory biology of crustaceans, he came to the University of Florida to continue his studies. After a brief hiatus to learn electrophysiological techniques at The Marine Biological Laboratory in Woods Hole, Massachusetts, he married Kathleen D. Huber and settled in St. Augustine to begin his research at The Whitney Laboratory. After another hiatus to The Marine Biological Laboratory as a Grass Fellow in the summer of 1988, he returned to complete the requirements for the degree of Doctor of Philosophy in zoology in May, 1989. He has accepted a postdoctoral position in the Section of Molecular Neurobiology at the Yale University Medical School in New Haven, Connecticut.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Zoology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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